The effect of abiotic and biotic variables on culturing conditions of Calanoid copepod *Acartia grani*
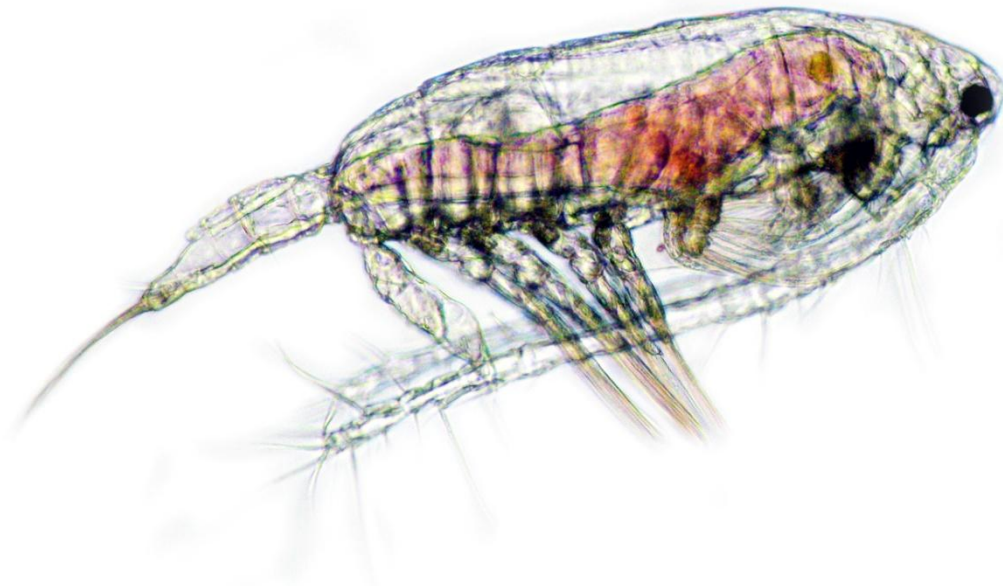
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The effect of abiotic and biotic variables on culturing conditions of Calanoid copepod *Acartia grani*

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Preface

The work described in this document was made between the months of November 2011 and September 2012, initially on IPIMAR - Algarve, and later at the Mariculture Center of Calheta (CMC), in Madeira Island. The work was organized in two phases: one was to acquire knowledge of microalgae and copepods in IPIMAR; and the second phase, performed at CMC facilities, was the performance of all the experiments that gave rise to this thesis.

Acknowledgements

Abstract

Calanoid copepods, incluinding species of the genus *Acartia* are commonly used for larval diets of marine finfish. The interest in copepods for marine fish aquaculture is growing especially in large-scale culturing. However, studies quantifying the optimal conditions for intensive copepod production are generally lacking for most species. The present thesis was conducted to evaluate the effect of abiotic and biotic variables on culturing condition of Calanoid copepod *Acartia grani*. The egg hatching rate (EHR) at different temperatures showed a clear trend of increasing EHR in warmer temperatures; 36 hours after incubation, 74.7% of eggs had hatched in the 28°C treatment (Chapter I). In Chapters II to Chapter V we analyzed the egg hatching rate (EHR), egg production (EP) and population growth of the different variables imposed. The highest egg hatching observed within each variable analyzed was: (1) 60%, 72h after incubation at 24°C within the temperature experiment (Chapter II) ; (2) the photoperiod experiment (Chapter III) 21%, 48h after incubation with light regimen of 6L:18D within the photoperiod experiment. (3) 44.2% at 72h in the Rho+T-Iso diet treatment (Chapter IV) and finally (4) 30.4%, 48 hours after incubation in the diet experiments using heterotroph *Oxyrris marina* (Chapter V).

Egg production results varied among variables and within each experiment best results were found for: 18°C, which had an EP of 10.5±1.5 eggs female^{-1}day^{-1}; photoperiod of 12Light:12Dark achieved an EP of 9.5±1.9 eggs female^{-1}day^{-1}; diet with *Rhodomonas marina* obtained 4.7±0.9 eggs female^{-1}day^{-1}; and finally in Chapter V, the diet *Rhodomonas marina* + *Oxyrrhis marina* had 3.7±0.6 eggs female^{-1}day^{-1}.

Regarding results of population growth when all developmental stages were included , we found that the temperature of 18°C produced a population increase from 12 to 663.0±52.2 individuals in 12 days (Chapter II); photoperiod 12Light:12Dark increased to a final population of 964.3±410.7 copepods. Last two experiments that evaluated the use of different diets revealed a population increase from 12 to 1783.0±560.6 individuals with the use of a binary diet composed by *Rhodomonas marina* + Tahitian strain of *Isochrysis sp.*; and in the last experiments (Chapter V) the best diet was once more, a binary diet composed by *Rhodomonas marina* and *Oxyrrhis marina* with a mean final population of 1795.0±451.8 copepods.

Cannibalism occurred in all tested densities (125; 250; 500; 1000; and 2000 ind./L) (Chapter VII) and conclusion was that 125ind./L induced significantly lower cannibalistic rates.

In Chapter VI we analysed the lipid and fatty acids composition of *Acarti grani* with different diets. The results show a moderate level of total lipids in the two samples. In the first diet (Rho) a total lipid content of 9.4±2.2% was found and for binary diet Rho+Oxy,

lipid content was of 8.6±0.1%. In the fatty acid composition no significance differences were observed (p>0.05). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were present in both samples in high amounts, witn a mean ratio of DHA/EPA of 2.29 for copepods fed on Rhodomonas and 2.18 for copepods fed Rhodomonas + Oxyrrhis.

In general, we concluded that the following parameters are a good choice for maximization of the culture of *A.grani*; Temperature: 24°C; Photoperiod: 12Light:12Dark; Diet: *Rhodomonas marina* + *Oxyrrhis marina*; Density: 150ind./L.

Resumo

Os copépodes Calanóides, em particular as espécies do género *Acartia* são geralmente usadas como dieta para larvas de peixes ósseos. O interesse nos copépodes em aquacultura de peixes marinhos tem aumentado, em especial na produção de culturas em grande escala. No entanto, para a maioria das espécies existem poucos estudos que refiram as condições óptimas de cultura. A presente tese foi realizada para avaliar o efeito de variáveis abióticas e bióticas nas condições de cultura do copépode Calanóide *Acartia grani*. As taxas de eclosão (EHR) em relação à temperatura mostraram que aumentavam com a subida da mesma; Após 36 horas de incubação, 74.7% dos ovos tinham eclodido no tratamento de 28°C. Do Capítulo II até ao Capítulo V analisamos as taxas de eclosão (EHR), a produção de ovos (EP) e o crescimento da população com as diferentes variáveis impostas. As maiores taxas de eclosão observadas entre as variáveis analisadas foram: (1) experiência da temperatura (Capítulo II) apresentou uma EHR de 60%, 72horas após incubação dos ovos a 24°C; (2) a experiência do fotoperíodo obteve uma EHR de 21%, 48h após incubação com um regime de 6Luz:18Escuro (Capítulo III); (3) na experiência das dietas (Capítulo IV) foi encontrada uma EHR de 44.2% às 72h na dieta Rho+T-Iso; (4) na experiência das dietas utilizando o organismo heterotrófico Oxyrrhis marina a maior taxa de eclosão foi verificada na dieta T-Iso (30.4%) 48h após a incubação (Capítulo V).

Os resultados da produção de ovos variaram com as diferentes variáveis e entre as experiências. Os melhores resultados foram: 18°C, com uma produção de 10.5±1.5 ovos fêmea^{-1}dia^{-1}; para o fotoperíodo de 12Luz:12Escuro a EP foi de 9.5±1.9 ovos fêmea^{-1}dia^{-1}; finalmente na dieta de Rhodomonas marina + Oxyrrhis marina, a EP máxima foi de 3.7±0.6 ovos fêmea^{-1}dia^{-1}.

Em relação aos resultados do crescimento da população, quando todas as fases foram consideradas (incluindo ovos), a temperatura de 18°C apresentou um crescimento dos 12 para 663.0±52.2 indivíduos em 12 dias (Capítulo II) e o fotoperíodo 12Luz:12Escuro teve uma população final de 964.3±410.7 copépodes. As últimas duas experiências em que foi analisado o papel das diferentes dietas, revelaram um aumento da população de 12 para 1783.0±560.6 indivíduos com o uso de uma dieta binária composta por *Rhodomonas marina* + Tahitian strain of *Isochrysis sp.* e na última experiência (Capítulo V) os melhores resultados foram uma vez mais com uma dieta binária composta por *Rhodomonas marina* e *Oxyrrhis marina*, onde foi verificado uma população média de 1795.0±451.8 copépodes, após 12 dias de cultura.

O canibalismo ocorreu em todas a densidades testadas (125; 250; 500; 1000; e 2000 ind./L) (Capítulo VII) e a conclusão foi que com 125ind./L as taxas de canibalismo eram significativamente inferiores.

No Capítulo VI foi analisado os lípidos e os ácidos gordos na composição da *Acartia grani* com diferentes dietas. Os resultados mostraram um teor moderado de lípidos totais nas duas amostras. Na primeira dieta (Rho) obteve-se um valor de lípidos totais de 9.4±2.2% e de 8.6±0.1% no caso da dieta binária Rho+Oxy. Na composição de ácidos gordos não foram observadas diferenças significativas ($p > 0.05$).

No geral concluímos que os seguintes parâmetros são uma boa opção para maximizar o cultivo do copépode *Acartia grani*; Temperatura: 24°C; Fotoperíodo: 12Luz:12Escuro; Dieta: *Rhodomonas marina* + *Oxyrrhis marina*; e uma Densidade: 150ind./L.

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Introduction

1| Introduction

1.1 Importance of Production of Live Feed in Aquaculture

Aquaculture is one of the fast growing food producing sectors in the world that plays an important role in satisfying the needs for seafood worldwide. In fact, in the period 1970-2008, the production of food fish from aquaculture increased at an average annual rate of 8.3 percent, while the world population grew at an average of 1.6 percent per year. The combined result of development in aquaculture worldwide and the expansion in global population is that the average annual *per capita* supply of food from aquaculture products for human consumption has increased by ten times, from 0.7 kg in 1970 to 7.8 kg in 2008, at an average rate of 6.6 percent *per* year (Grainger, 2010).

Despite the increased production over the years, aquaculture production, particularly fish farming, will always depend on "seed" supplying of known quantity and quality at any time (Andrade et al., 2012). Once spawning of a fish species is under control larviculture protocols have to be established in order to meet the biological and nutritional demands of the larvae.

The main bottleneck for fry production of many marine fish species is related to the high mortality rates associated with larval first feeding. Many finfish larvae rely on live prey for several reasons. Prey size may affect the prey ingestion by early fish larval (Planas and Cunha, 1999). The larvae of numerous marine fish require small prey (about 50–100μm wide) at first feeding (Detwyler and Houde, 1970; Yúfera and Pascual, 1984) due to the small size of their mouth. In many cases, even the commonly used rotifer *Brachionus rotundiformis*, type ''S'', may be too large (Houde, 1973; May et al., 1974; Doi and Singhagraiwan, 1993). Moreover, moving prey seems to be more attractive to fish larvae than inert particles, which have a tendency for sinking or agglomerating, making it unavailable for a quick consumption. Moreover, the gut of many fish larvae (particularly altricial larvae) is not fully developed at the time of first feeding, and it is an advantage to get live prey which brings some exo-enzymes to the fish larvae (Conceição et al., 2010).

High larval mortalities are also often associated to the lack of a nutritionally adequate diet (Nanton and Castell 1998). Major gap in knowledge of fish larval nutritional requirements still remains (Conceição et al., 2010). Nevertheless, it has become evident that fish larvae have specific nutritional requirements that can be linked to the biochemical characteristics of their natural prey. In most aquaculture enterprises, fish and crustacean larvae are reared using rotifers and *Artemia* nauplii as live prey. The rotifer, *Brachionus plicatilis* constitute an essential part of the feeding during the larval stages of marine fish and crustaceans. Its body size; high growth rate; good tolerance to culture conditions or handling and feeding by filtration of particles in suspension make this organism an appropriate prey to start feeding after reabsortion of vitelline reserves. The ready

availability of *Artemia* nauplii, through the purchase of cysts and subsequent hatching of their nauplii (Lavens and Sorgeloos, 2000) makes them the most convenient and least labour intensive live food available for aquaculture (Lavens and Sorgeloos, 2000). Metanauplius I (instar II) of *Artemia* are continuous non-selective filter feeding organisms, just as rotifers, which is an important characteristic and the basis of the bioencapsulation processes (Van Stappen, 1996). Despite significant research and development has been achieved on enhancing culture techniques or enrichment methods to improve the availability and nutritional value of rotifers (Rainuzzo et al., 1997; Sargent et al., 1997) and *Artemia* nauplii, the use of these live prey does not always lead to optimal larval growth, as they usually have an inadequate fatty acid profile (Olivotto et al., 2008a,b). Dietary lipids are recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe et al. 1983). In particular, the importance of *n*-3 HUFA for marine fish larvae has been widely studied and their requirements have been reported for some species (Planas and Cunha, 1999).

Alternatives to rotifer culture have been used such as chicken egg yolk, frozen abalone larvae, copepods, processed krill and oyster ovaries (Koga and Naghama, 1981; Nagahama and Hidaka, 1982), but results have been unsuccessful on a large scale due to improper nutrition or difficulty in supplying the quantity of food required and when needed (Koga and Naghama, 1981). Despite recent progress in the development of inert diets for fish larvae (e.g., Lazo et al., 2000; Cahu and Infante, 2001; Koven et al., 2001), feeding of most species of interest for aquaculture still relies on live feeds during the early life stages. Even the 'Artemia replacement' products increasingly used in commercial operations are normally used in co-feeding with live feeds.

For all the above, the selection of an adequate live food for larvae is considered as a key factor for the future expansion of marine aquaculture, unless breakthroughs in the development of microdiets for marine fish larvae are introduced as a viable alternative to live feed (Liu and Xu, 2009). Thus, the availability of live prey of high nutritional value for maximal growth and survival of fish and crustacean larvae is of fundamental importance (Drillet et al., 2008; Fleeger, 2005; Olivotto et al., 2008a,b; Sargent et al., 1997). Among these potential alternatives, copepods are the best prospective candidate and the development of many species is increasing their potential as live prey.

1.2 Copepods

1.2.1 Copepods and their use in aquaculture

The name copepod is derived from the Greek *kope* meaning 'oar' and *podos* meaning 'foot', and refers to the flat, paddle-like swimming legs. Around 200 families with some 1650 genera and 11.500 species were classified by 1993 (Humes, 1994).

Copepods are aquatic animals, mostly marine, although many species occupy freshwater or estuarine habitats. In nature, they constitute a first vital link in the marine food chain leading from primary producers to fish. They can represent up to 80% of the zooplankton biomass in the water column (Mauchline, 1998). In the open water marine environment, calanoids dominate the herbivorous zooplankton and provide the food-chain base for practically all marine fish larvae and planktivorous fish (Pauly and Christensen 1995).

The diets of pelagic copepods are characteristically broad (Kleppel, 1993) and strict herbivory copepods rarely exists in nature. Most copepods prefer feeding on microzooplankton due to their large size, easy perception, as well as the relatively high food quality (Batten et al., 2001; Gifford et al., 2007; Campbell et al., 2009). Moreover, copepods are capable of switching their feeding behavior depending on the prey composition, and particularly on the relative abundance of phytoplankton and microzooplankton (Landry, 1981).

It is well accepted that many copepods are a valuable source of food for fish larval rearing although they are not often used in aquaculture industry. Interest in the use of copepods in aquaculture has grown since the 1980s (Schipp, 2006). Over the past few years there have been several review articles published and numerous conferences, conference sessions and workshops dedicated to discussions of copepod culture and the important role that copepods can play as feeds for marine fish larviculture (Bell et al., 1997; Støttrup, 2000; Kleppel and Hazzard, 2002; Lee et al., 2005). The three main free-living copepod orders, Cyclopoida, Harpacticoida and Calanoida have each been investigated for their suitability as feeds for larval and juvenile fish (Marcus, 2005; Gaudy and Guerin, 1982; Ogle et al., 2005; Payne and Rippingale, 2001a; Phelps et al., 2005; Schipp et al., 1999; Støttrup and Norsker, 1997; Sun and Fleeger, 1995).

Cyclopoids include pelagic, epibenthic, benthic and parasitic species and inhabit both freshwater and marine environments, but most abundant in freshwater. Cyclopoid copepods with obligatory pelagic nauplii are pelagic and are used occasionally in aquaculture and densities of ~5000 ind./L are possible to achieve in cultures (Phelps et al., 2005; Su et al., 2005). Cyclopoids definitely offer a great potential for aquaculture and aquarium trades but they have not been intensively studied maybe because of the

difficulties in harvesting nauplii from culture (Støttrup, 2006) and lack of storage possibilities for eggs.

Harpacticoids include over 50% of copepod species and are primarily marine free living, benthic organisms. Harpacticoid copepods can be produced in high density cultures, but their benthic habitat may limit their availability to pelagic fish larvae (Drillet et al., 2008; Lee et al., 2006; Olivotto et al., 2008b; Puello-Cruz et al., 2009; Støttrup, 2006). Fleeger (2005) cites the advantages of using harpacticoid copepods in marine fish cultures. Amongst them, according to Lavens and Sorgeloos (1996), benthic harpacticoid copepods of the genera Tisbe and Tigriopus have characteristics that facilitate cultivation, such as high fecundity, short generation time, tolerance to extreme environmental changes (salinities ranging from 15 to 70psu and temperatures from 17 to 30°C), acceptance of a variety of food sources (microalgae, yeast, dry feeds) and high population densities can be achieved.

Calanoids are predominantly pelagic, occurring at all depths, with some living near bottom benthic species. They are selective feeders, feeding on small phytoplankton cells by filtration or predators feeding on animal prey including copepod eggs. Several species belonging to the calanoida order have been proven as ideal food for many cultured marine larvae (Molejón and Alvarez-Lajonchère, 2003; Marcus, 2005; Schipp, 2006; Milione and Zeng, 2008; Camus and Zeng, 2009) (Table I), showing excellent nutritional value when compared to rotifers and *Artemia*.

Table I - Species of Calanoids used in aquaculture as live prey for marine fish species (Støttrup, 2003).

Specie	Origin or application	Marine fish species	References
Acartia longiremis	Collected from wild	Wolf fish, Anarhichas lupus	Wolf fish Ringø et al. (1987)
Acartia pacifica and *A. plumose*	Collected from wild	Asian sea bass, Lates calcarifer; grouper Epinephelus fuscoguttatus	Sunyoto et al. (1995)
Acartia sinjiensis		Red snapper, Lutjanus argentimaculatus	Doi et al. (1997b)
Acartia tonsa		Cod, Gadus morhua	Jung & Clemmesen (1997)a
Acartia tonsa	Laboratory culture	Fundulus spp., Elops saurus and squid	Turk et al. (1982)
Acartia spp.		Golden snapper, Lutjanus johnii, Loligo pealie Mangrove jack, Lutjanus argentimaculatus	Schipp et al. (1999)
Eurytemora affinis		Striped bass, Morone saxatilis	Chesney (1989), Tsai (1991)
Eurytemora affinis and *Acartia tonsa*	Outdoor tanks	Turbot, Scophthalmus maximus	Kuhlmann et al. (1981)
Gladioferens imparipes		Dolphin fish, Coryphaena hippurus Sea horse, Hippocampus angustus	Rippingale & MacShane (1991)
Gladioferens imparipes	Co-fed with rotifers	Australian dhufish, Glaucosoma hebraicum Pink snapper, Pagrus aurata	Payne et al. (2001)a
Metridia longa	Collected from wild	Wolf fish, Anarhichas lupus	Ringø et al. (1987)
Temora longicornis		Halibut, Hippoglossus hippoglossus	Rønnestad et al. (1998)

Moreover, calanoid copepods are also interesting due to their pelagic, produce resting eggs. However, many calanoids cannot be kept at high densities without negative

side effects, like a decrease in hatching success or high mortalities (Jepsen et al., 2007; Peck and Holste, 2006).

Independently of the copepod order (Calanoida, Harpacticoida or Cyclopoida), it is well known that raised copepods as well as harvested zooplankton contain biochemical characteristics which makes them a good alternative or supplement of live prey for larval rearing (Naess et al., 1995; Shansudin et al., 1997; Støttrup and Norsker, 1997; McEvoy et al., 1998; Rønnestad et al., 1998; Payne and Rippingale, 2000; Støttrup, 2000; Payne et al., 2001; Evjemo et al., 2003). They are considered to be "nutritionally superior live feeds" for commercially important cultivable species, as they are a valuable source of protein, carbohydrates, enzymes (amylase, protease, exonuclease and esterase) and lipid (with particular regard to fatty acids) (Watanabe, 1979; Watanabe et al., 1983; Witt et al., 1984; Shansudin et al., 1997; Toledo et al., 1999), which are essential for larval survival, growth, digestion and metamorphosis (Støttrup, 2000; Molejón and Alvarez-Lajonchère, 2003; Kleppel et al., 2005).

The fact that these organisms contain highly unsaturated fatty acids, especially *n-3* fatty acids, in comparison to other live prey used in aquaculture is an advantage, not only because fatty acids are important components of biomembranes in fish, as well as providing energy (Cowey and Sargent, 1972; Rodriguez et al., 1993; Izquierdo, 1996; Barclay and Zeller, 1996; Sargent et al., 1997), but also because there is likely no need for further enrichment, as is required with rotifers and *Artemia* nauplii. Sargent et al. (1997) listed the major advantages in using copepods for marine finfish larval feeds as follows:

(1) A preponderance of phospholipids rather than triacylglycerols;

(2) Levels and ratios of fatty acids that more closely approximate the natural diet of marine finfish larvae;

(3) The probability of optimal protection of polyunsaturated fatty acids (PUFA) by natural antioxidants against peroxidation and the delivery of optimal levels of natural antioxidants to the larvae.

In addition, copepods are also known to have greater digestibility (Schipp et al., 1999) because of a slower passage through the gut of fish larval than *Artemia* spp., which leads to a more complete digestion and efficient nutrient uptake (Pedersen, 1984). This may be due to the fact that copepods have higher digestive enzyme contents than *Artemia* which can be used by the fish larvae as exo-enzymes (Munilla-Moran et al., 1990). Finally, the wide range of body sizes of copepods makes them more suitable to predation by larvae and juvenile fish.

Støttrup (2000) and Payne et al. (2001) strongly suggest that the inclusion of copepods in the aquaculture industry may increase the number of successfully reared fish species.

The future expansion of marine aquaculture may further encourage work on copepods towards the development of reliable production systems or, alternatively, the production of resting or diapause eggs for sale on a commercial scale (Støttrup, 2003).

1.2.2 Considerations of copepod culture

Despite significant progress in copepod cultivation methods (Payne and Rippingale, 2001b; Støttrup, 2003; *Lee* et al., 2005), establishing cost-effective protocols for mass production are still a challenge. The progress towards mass-culture techniques of copepods has been slow and not thoroughly researched in larviculture. In addition, development of this field has been fragmentary (Støttrup, 2000). Intensive research is therefore needed in copepod culture engineering to enhance the appropriate culture techniques that will promote the feasibility and cost-effectiveness that allow their in the aquaculture industry (Ajiboye et al., 2011).

1.2.3 Factors affecting production

The most important factors controlling stage duration of copepods are temperature, light, food quantity and quality (Cook et al., 2007), amongst others. The primary need for information with regards to large-scale and dense copepod cultures resides in the effects of these parameters during intensive cultivation.

Temperature has to be adapted to each population of copepods and is often analogous to the conditions the population is facing in wild because both populations are adapted to these particular environments. Species inhabiting coastal environments are usually more tolerant to variations in salinity and have wider thermal tolerance. Temperature has been positively related to growth rate (MacLaren, 1965-1966; Landry, 1975) and egg production rate (Uye, 1981), but inversely related to body size (Deevey, 1960).

Light rhythm is another important factor controlling the physiological performances of copepods, activating sometimes the production of diapause stages (Marcus, 1982; Alekseev et al., 2007). This factor affects egg production and hatching success of *Acartia sp.* cultures; longer light exposure increased the 48 hour hatching success of the eggs

(Camus and Zeng, 2008; Peck et al., 2008). However, cultivation procedures kept constant over time under particular conditions are likely to select for specific traits. For example, cultures of *A. tonsa* from the Danish Technical University (DTU-Aqua) have lost their diel feeding and egg production rhythm due to excess food availability and absence of predators through 150 generations (Tiselius et al., 1995).

The amount and quality of food are also essential parameters that enhance the production of copepod cultures. Effects of food quantity and quality have been assessed in natural populations because of their importance at the ecologiical level. Most of the information present in the literature can be extrapolated and used for culture purposes. Quantity-wise, all copepods do not have the same need to reach their maximum growth and production potentials even when closely related (Jonasdottir, 1989). Too little food tends to slow down stage development, increase the competition for food between the individuals, and ultimately increases mortality (Berggreen et al., 1988). Food quality should be adjusted for the target number of copepods, and the size of the prey should be consistently adapted not only to the species but also to the development stages that need to be fed (Berggreen et al., 1988; Hansen et al., 1994). Previous research has shown that a mixture of different diets generally enhances the somatic growth and egg production of copepods in the laboratory (Harris, 1977; Jonasdottir, 1994; Klein Breteler, 1980), but also the delivery of right food item at the right development stage increases the overall success (Koski et al., 2006; Murray and Marcus, 2002). The food items should be adapted to the copepod feeding habits for example whether the copepods raised are raptorial feeders or suspension feeders.

1.2.4 Culture of Calanoids – Family *Acartiidae*

The most frequently cultured calanoid species belong to the genera found in coastal waters, such as those of the genera *Acartia*, *Centropages*, *Eurytemora* and *Temora*. These copepods are small, with relatively short generation times and a wide thermal and salinity tolerance (Støttrup, 2003).

On this work, we focused in one copepod of the family *Acartiidae* because they are common in coastal and estuarine habitats in all oceans of the world. They are thought to be mainly adapted to high food concentrations which are found in estuaries and upwelled waters. Their wide distribution in space and time may be due to the fact that a number of *Acartia* species are known (1) to produce resting eggs which lie dormant in the sediment and allow them to appear suddenly in the plankton when conditions are favorable (e.g. Uye, 1983; Lindley, 1990; Naess, 1991; Belmonte, 1992) and (2) to be transported in

ships ballast water to other parts of the world (Hirakawa 1988). The keeping of these copepods alive in the laboratory is undoubtedly of great interest to obtain better information on their biology.

Because they are the principal link in the marine food chain in some areas, there are many studies about growth in copepods of the genus *Acartia* (Paffenhöfer, 1970; Durbin and Durbin, 1978; Landry 1978; Klein Breteler and Gonzalez, 1982; Kimmerer and Mckinnon, 1987; Berggreen et al., 1988; Escribano and McLaren, 1992; Huntley and Lopez, 1992; Tsuda, 1994; Saiz et al., 1998; Campbell et al., 2001; Hirst and Kiørboe, 2002; Leandro et al., 2006). However, growth in the early developmental stages has rarely been described (Durbin and Durbin, 1978; Berggreen et al., 1988; Calbet and Alcaraz, 1997; Leandro et al., 2006).

1.2.5 The Copepod *Acartia grani*

Since the *Acartia* species can be propagated in tanks and ponds, the possibility of their use as food organisms for fish larvae has been proposed by several authors (Ikeda, 1973; Omori, 1973). In South East Asia nauplii of the copepod genus *Acartia* (Calanoida: *Acartiidae*) have been most commonly used because adults of this genus are easily captured by light attraction at night.

Among the calanoids, *Acartia grani* (Sars G.O. 1904) (Table II), is a small calanoid copepod typical of coastal, semi-confined ecosystems, conditioned by a high degree of instability in physical (temperature) and biological (food) conditions (Calbet *and Alcaraz,* 1996). *Acartia grani* has been recorded in different areas of the Atlantic Ocean, such as Canary Islands (Corral, 1970; Vives, 1982), in the Western Mediterranean Sea (Guerrero and Rodríguez, 1998, Saiz et al., 1998), in the Portuguese coast (Vilela 1972) and also in the Madeira Archipelago (http://copepodes.obs-banyuls.fr).

This is a calanoid species that present good potential for use as live feed in aquaculture (Cunha et al., 2007), because it is considered to be one of the most abundant in the oceanic systems and also because they are an important component in the diet of a great quantity of plankton species.

Table II- Taxonomic classification of the copepod *Acarti grani*.

Phylum	Arthropoda
Subphylum	Crustacea
Class	Maxillopoda
Subclass	Copepod
Order	Calanoida
Superfamily	Diaptomoidea
Family	Acartiidae
Genus	Acartia
Specie	*Acartia grani* (G.O. Sars,1904)

1.2.6 Biology *Acartia grani*

A. grani is small calanoid copepod typical of coastal, adults have approximately 1000µm in length, and their N1 nauplii are approximately 70µm in length. *Acartia* eggs (Fig.6A) are 70-80µm in diameter, spherical, covered with short spines and are slightly heavier than seawater. At 25°C, most eggs hatch too nauplii within 48 h. The nauplii hatching from these eggs are very small and can be used directly to feed the fish larvae since they are naturally rich in highly unsaturated fatty acids (HUFA's).

Nauplii (Fig.6A) progress through six stages, (N1 through N6) to become copepodites (Fig.6D), which then progress through six stages (C1 through C6) to become sexually mature adults (Fig.6E and F) (Marcus and Wilcox, 2007).
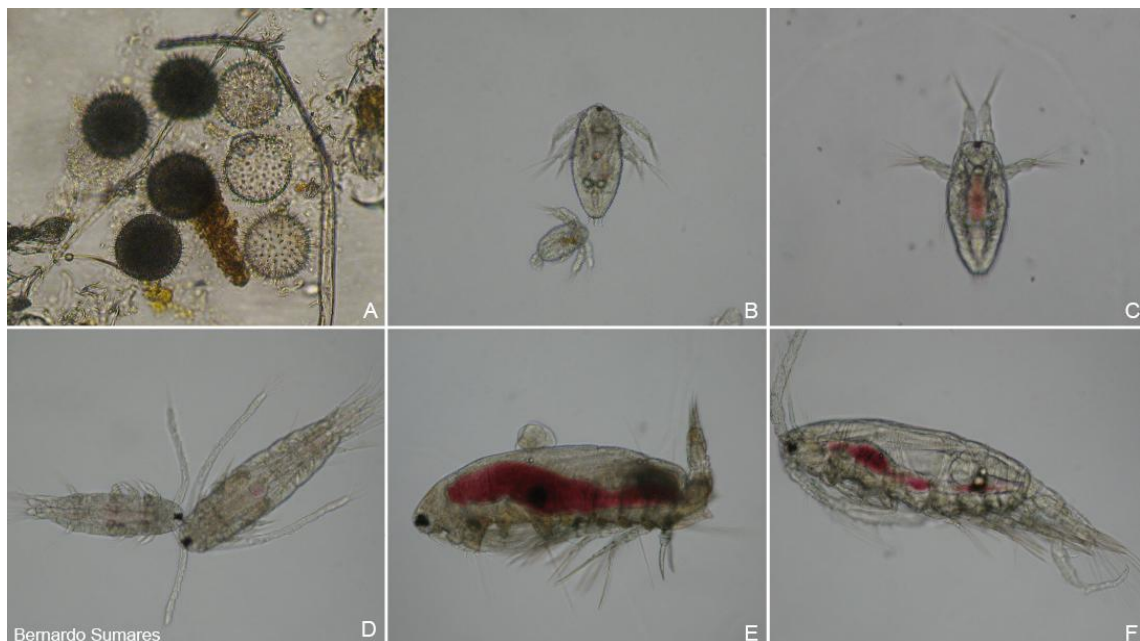
Figure 1 - *Acartia grani* development. (A) Viable eggs dark; (B) and (C) Three different stages of nauplii; (D) Two stages of copepodites; (E) Adult female; (F) Adult male.

Both *A. grani* male and females are characterized by a fragmented area, but the internal area is provided with loop-shaped seminal ducts opening directly in the proximal zone of the egg-laying duct. This organization, at least for the external area with lateroposterior genital slits (http://copepodes.obs-banyuls.fr). In Figure 7 is possible to see external morphology and relevant terminology of *Acartia grani*.

Adult males and females are visually distinguished by conformation of their antennae, urosome, and swimmerets (Sabatini, 1990). Males use their antennae to clasp a female to allow them to deposit a spermatophore on the female urosome for fertilization. Males have shorter lives than the females (Parrish and Wilson 1978, Sazhina, 1987) which has an impact on egg viability in maturing batch cultures.

A. grani are broadcast spawners, and do not carry their eggs as some copepods do, which allows for easier egg collection and storage (Marcus and Wilcox, 2007).

Acartia primarily feeds on phytoplankton, but also consumes ciliates, rotifers and their own eggs and nauplii (Mauchline, 1998). They use their mouthparts to create water currents that bring food particles towards the copepod. Consumable prey size ranges from 5μm to 100μm (Petipa, 1959). Smaller particles are then captured passively and are directed towards the mouth by setae.
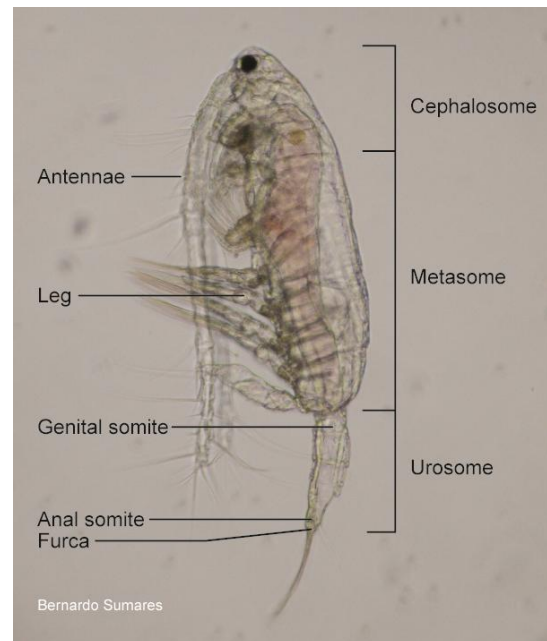
Figure 2 - External morphology and relevant terminology of *Acartia grani*.

1.2.7. Parental Culture of *Acartia grani*

Before starting a culture of *A.grani* it is important to remember, that copepods are fragile organisms and when subjected to rough treatment, vigorous aeration or shifted to other tanks, they could eventually die or lose some important appendixes that are vital for completion of the life cycle. Therefore, acclimatization processes must be slow and gentle.

Copepod eggs used in this thesis were collected in Algarve during the winter of 2010 and were brought to CMC facilities in December of 2011. Eggs were maintained at 4ºC in the dark. In February 2012 first attempts of *Acartia grani* production were done at CMC experimental facilities.

Parental culture was started in a clean 500L tank, with filtered, UV-treated seawater (38 psu) (Fig.3A). Approximately 250.000 storage eggs (Fig.3B) were placed in the culture tank (Fig.3C and Fig.3D), without aeration in the first 24h and continuous light (24L:0D) to promote the hatching of the nauplii.

Forty eight hours later the algae, *Rhodomonas marina* was supplied at a concentration of 1500 µgCL^{-1}. A main factor in the production of this species is the quality and amount of microalgae supplied. Phytoplankton must have good quality, free of contamination, otherwise the copepods will not consume and the contaminated microalgae will deteriorate the water quality. If the quantity of food is high, *A.grani* will not consume all and the same problem will arise, occurring the "crash" of copepod culture. On the other hand, lack of microalgae, will promote cannibalism. Cleaning of the tank bottom was done every 2 days, in order to maintain water quality. For description of *R. marina* production see chapter 1.3.2.

First trials revealed that 9-12 days after incubation, the cycle was completed, meaning that occurrence of egg production was noticed (Fig.3E). Egg collection was performed with a squeeze siphon (Fig.3F) and the help of two sieves: a 200µm sieve for collection of the adults and copepodites, the 55µm to collect the eggs and nauplii. Collected eggs were either stored in the dark at 4ºC or were used to initiate new cultures.
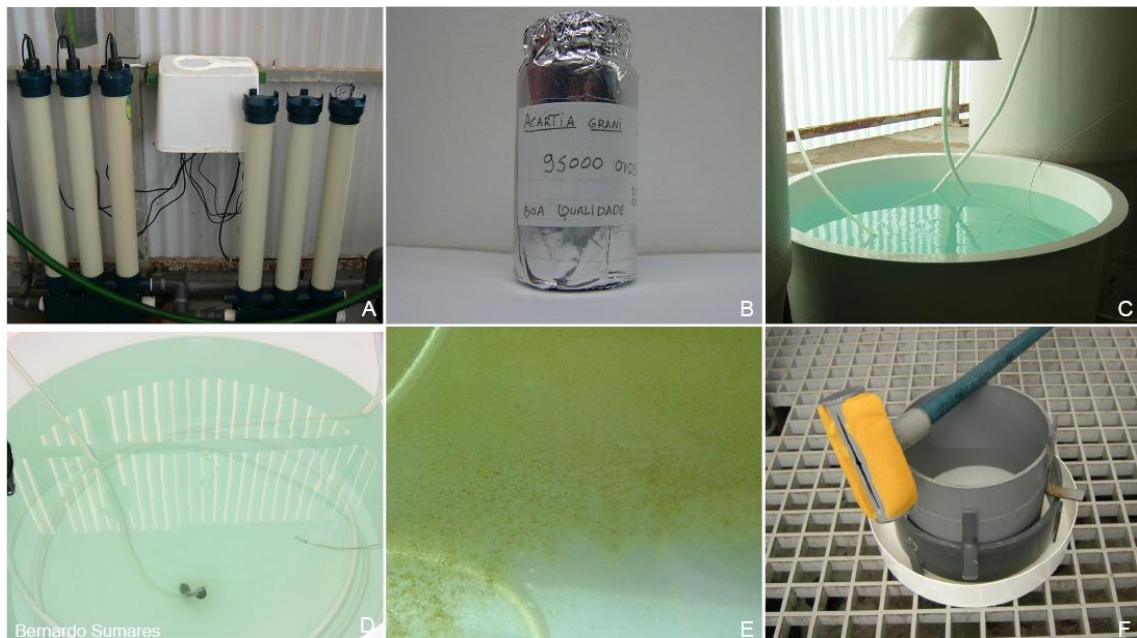
Figure 3 - Parental Culture of *Acartia grani* in 500L tank. (A) Filter set and ultraviolet sterilizer; (B) Stored eggs of *Acartia grani* in freeze at 4°C, ready to use; (C) Production tank (500L); (D) Production tank with the first eggs in the bottom; (E) Eggs and organic matter in the bottom of tank; (F) Cleaning and collecting system of eggs: two sieves 200 and 55µm and squeegee siphon.

1.3 Microalgae

Ocean phytoplankton, with a production of several hundred billion tonnes of dry weight *per* year (Pauly and Christensen, 1995), forms the base of the aquatic food chain, contributing to the production of some 100 million tonnes of renewable resources *per* year from fishing. The first microalgae species used in aquaculture have been selected from those that developed naturally in the marine environment of pioneering aquaculture farms and were probably the easiest ones to cultivate (Muller-Feuga et al., 2003).

Microalgae must possess a number of key attributes to be useful aquaculture species. They must be of an appropriate size for ingestion, e.g. from 1 to 15µm for filter feeders; 10 to 100µm for grazers (Webb and Chu, 1983; Jeffrey et al., 1992; Kawamura, et al., 1998) and readily digested (Brown, 2002) Not all algal species are equally successful in supporting the growth and survival of a particular filter-feeding animal. Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for the feeding animal. Although there are marked differences in the compositions of the micro-algal classes and species, protein is always the major organic constituent, followed usually by lipid and then by carbohydrate (Coutteau, 1996)

In this work *Rhodomonas marina* was chosen as the main algae for feeding copepods (Fig.4), because this species has been indicated and used as an adequate alga for production with *Acartia grani* (Calbet and Alcaraz, 1997; Costa and Fernández, 2002; Rivero, 2008) because of the nutritional content, rich in proteins and lipids*.* However, reliance upon *Rhodomonas* can be problematic because it is relatively unstable in culture and is prone to suddenly dying off (Knuckey et al., 2005).

Figure 4 - General aspect of *Acartia grani* fed with *Rhodomonas marina*. (B) *Rhodomonas* cells inside the intestine.

1.3.1 Biology of *Rhodomonas marina*

This Cryptophyceae (Table III) is a flagellate unicellular red alga with a cell diameter between 9.2 and 9.9 µm (Fig.5). Cells appear red due to the phycoerythrin what dominates chlorophyll and takes up the blue-green light and emit red-orange light. This marine microalgae has been used as food for marine copepods (Jónasdóttir, 1994), oyster larvae and spat (Brown et al., 1998; McCausland et al., 1999; Muller- Feuga et al., 2003) and queen conch larvae (Aldana-Arana and Patiño-Suárez, 1998).

Table III - Taxonomic classification of *Rhodomonas marina*.

Domain	Eucaryote
Kingdom	Chromalveolata
Phylum	Cryptophyte
Class	Cryptophyceae
Order	Pyrenomonadales
Family	Pyrenomonodaceae
Genus	*Rhodomonas*
Species	*Rhodomonas marina* (P.A.Dangeard) Lemmermann
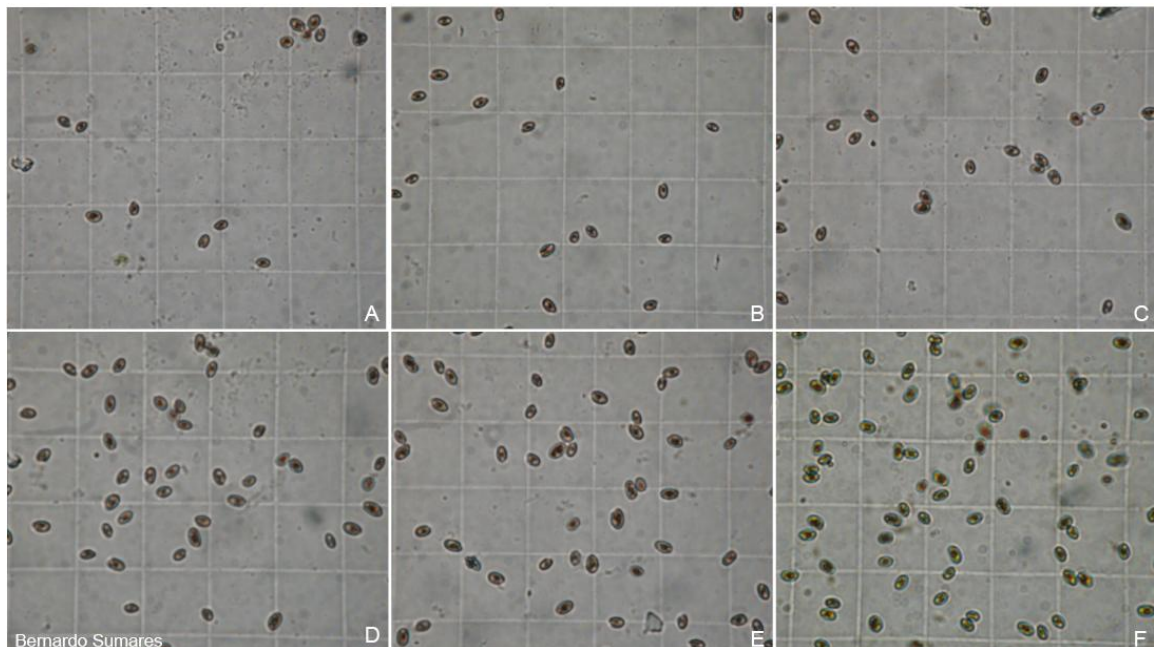
Figure 5 - The culture of *Rhodomonas marina* in different stages of maturation, in Malassez counting chamber. Objective magnification 40x.

1.3.2 Starter Culture of *Rhodomonas marina*

Microalgae sterile starter cultures were supplied by the Institute Investigation of fisheries and sea (IPIMAR - Algarve). The culture began in test tubes with seawater filtered and UV- treated 25 psu (Fig.6A).

From November 2011 to January 2012, several culture tests were performed in order to successfully cultivate *Rhodomonas marina* CMC facilities. In our first attempts, the culture "crashed" after 2-3 days or was contaminated with different kind of ciliates, which made difficult to proceed with the batch method. This algae is technically more demanding to culture than other algae traditionally used in aquaculture facilities (Knuckey et al., 2005). After some attempts, we successfully cultivated *Rhodomonas* in our facilities (Fig. 5), and used it as live feed to *Acartia grani*.
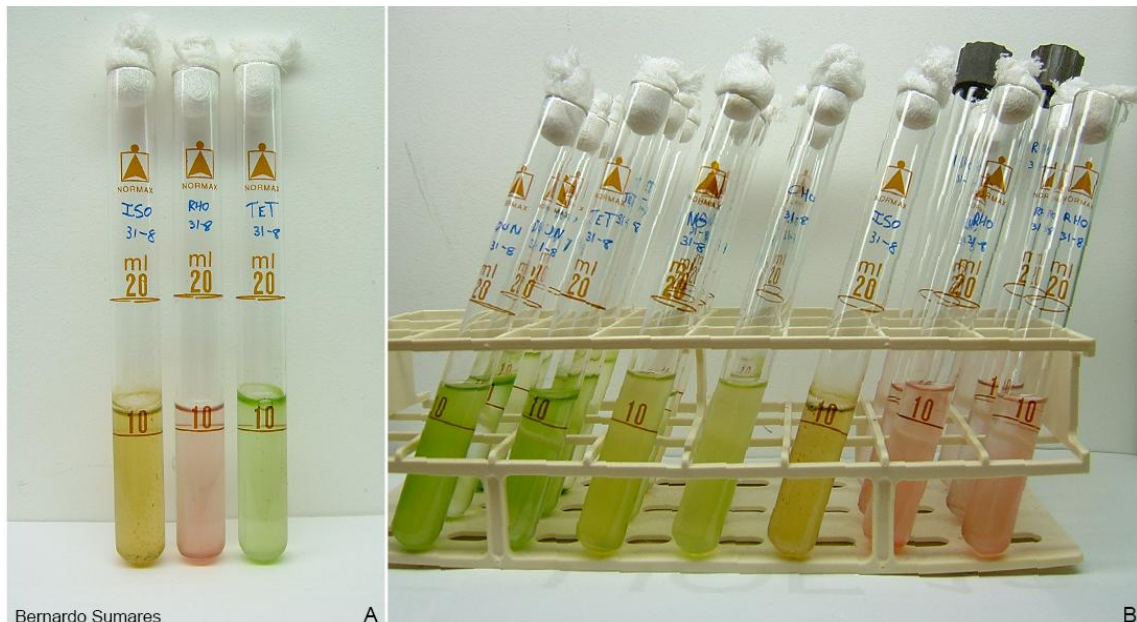
Figure 6 - Microalgae sterile starter cultures. (A) The three main algae used in this thesis; *Isochrysis sp.* (T-Iso), *Rhodomonas marina* and *Tetraselmis suecica* respectively. (B) The stock culture.

1.3.3 Algae Production

Microalgal species can vary significantly in their nutritional value, and this may also change under different culture conditions (Enright et al., 1986; Brown et al., 1997). Nevertheless, a carefully selected mixture of microalgae can offer an excellent nutritional package for larval animals, either directly or indirectly (through enrichment of zooplankton) (Brown, 2002). The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature (Table IV).

Table IV - Culture condition of microalgae.

Species	*Rhodomonas marina* *Tetraselmis suecica* *Isochrysis sp. (T-Iso)*
Temperature	20±1°C
Salinity	25 psu
Photoperiod	24 hours light
Aeration	Moderately
Nutrient	Nutribloom 1:1000ml

Figure 7 - Different microalgae and *Oxyrrhis marina*, in our laboratory, with controlled temperature (20±1°C).

The most optimal parameters as well as the tolerated ranges are species specific. All microalgae used in this work (Fig.6B and Fig.7) were produced according to the batch methodology. A batch culture is a discontinuous culture, sterilized and inoculated by the microorganism wanted. It enables its growth until nutrients become limited. The medium has to contain at least one energy, carbon, nitrogen and mineral ions source. A single inoculation of cells into a container (10-6000ml) of fertilized seawater (filtered and UV-treated) followed by a growing period of several days 5-7 and finally harvesting when the algal population reaches its maximum or near-maximum density $20 - 40 \times 10^5$ cel.ml^{-1}, depending the microalgae used. In practice, algae are transferred to larger culture volumes (Fig.8) prior to reaching the stationary phase and the larger culture volumes are then brought to a maximum density and harvested.
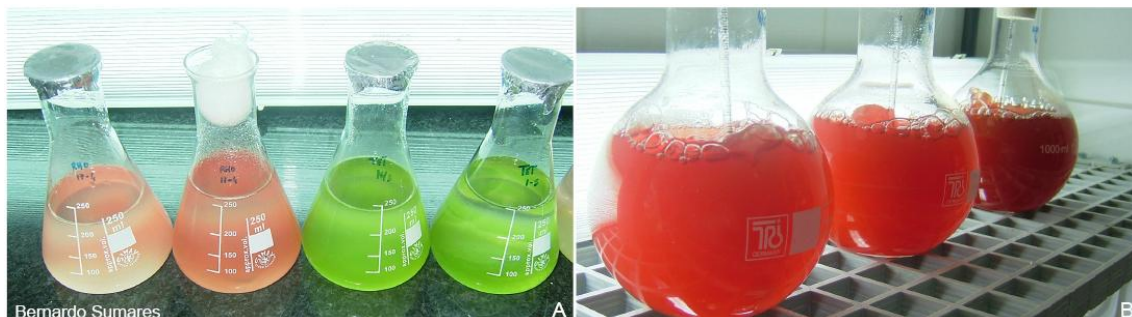
Figure 8 - Production of microalgae in our laboratory. (A) Erlenmeyer (250ml) with *Rhodomonas marina* and *Tetraselmis suecica*. (B) *Rhodomonas marina* in 1L balloons.

2| Objectives

2.1 Aim of the study

Numerous studies have demonstrated that copepods may have a higher nutritional value than *Artemia*, as the nutritional profile of copepods appear to match better the nutritional requirements of marine fish larvae. Furthermore, they can be administered under different forms, either as nauplii or copepodites at start feeding and as on grown copepods until weaning (Delbare, et al., 1996). Over the years several studies have focused in copepods culture, to understand the optimal biotic and abiotic factores that would satisfy the need of regular culture production.

Most *Acartia grani* studies published to date do not cover important biological aspects of culture, including optimal temperature and photoperiod conditions or best diet. Some of these parameters have however been determined for other species of copepods.

The aim of this thesis was to improve knowledge of the copepod *Acartia grani*, in order to determine the culture requirements and the most appropriate biotic and abiotic factores. This project was made in lab scale, but some of the culture conditions and requirements can be applied to large scale production.

The thesis presents seven chapters, excluding the introduction, discussion-final remarks and each chapter is briefly described below:

- To begin a copepod culture, is crucial to know the hatching rates (present in **Chapter I**), in order to prepare the correct food concentration, for supplying at the beginning of feeding stage.

- Variability in temperature characterizes seasonal succession and directly affects copepod reproduction and development (Ianora et al., 1992). In **Chapter II** different temperatures were tested to evaluate the optimal culture temperature.

- Photoperiod is one of the most significant cues for seasonality in nature (Hairston and Kearns, 1995) and could therefore be a key factor controlling female copepod reproductive status and population dynamics. This abiotic factor was investigated in **Chapter III**.

- Food quality and quantity are probably the most important factors regulating the productivity of copepod culture: **Chapter IV** and **V** tested different diets to determine the most appropriate diet for *A. grani* based on the most common cultured microalgae species.

- Copepods are a rich source of phospholipids, essential highly unsaturated fatty acids (HUFA), natural antioxidants and other essential compounds (Kraul et al., 1992; Sargent et al., 1997) **Chapter VI** describes the methodology used to identify the nutritional value of *Acartia grani*, based on lipid and fatty acids of adult copepod fed with *Rhodomonas marina* and *Oxyrrhis marina*.

- High densities are considered difficult due to density-related stress factores (Jepsen et al., 2007). One of the stress factores of high densities is cannibalism. In **Chapter VII** we tested this factor at different culture densities.

3| Chapters

Egg hatching success at different temperatures of calanoid Copepod *Acartia grani*

1. Introduction

Copepods are primary consumers in the oceans and are perhaps the most numerous metazoans on earth (Ohman and Hirche, 2001). Calanoid copepods play a key role in the cycling of nutrients and energy in marine ecosystems by forming a trophodynamic link between primary (phytoplankton) and tertiary (e.g. planktivorous fish) production (De-Young, 2004). The widespread distribution and abundance of members of this group partially result from adaptation of life history traits to match specific environmental (physical or chemical) conditions or constraints. Amongst these environmental conditions, temperature is often considered as the key external factor that affects life history traits and the population dynamics of copepods.

Variability in temperature characterizes seasonal succession and directly affects copepod reproduction and development (Ianora et al., 1992). Changes in species abundance in the Baltic sea, throughout the year, have been related to wide range variations in salinity and temperature that exceed those of the preferred niche of the calanoid species found there (Holste and Peck, 2006). Egg hatching rate and success are also reported to be temperature dependent (McLaren, 1966; Uye and Fleminger, 1976; Ban, 1994; Holste and Peck, 2005). Furthermore, temperature dependent hatching patterns are related to spawning temperature (Landry, 1975; Uye and Fleminger, 1976). Unfortunately, the functional response of reproductive success (i.e., egg production and hatching) to temperature variations in many calanoid species is not well known, having been studied in only a handful of species such as *Eurytemora affinis* (Gonzalez and Bradley, 1994) and a number of *Acartia* congeners (Tester and Turner 1991; Chinnery and Williams, 2004). Koski and Kuosa (1999) found that unfavorable temperature might be the probable reason for low hatching in *Acartia bifilosa* and Milione and Zeng (2008) found that temperature significantly affected hatching times of *Acartia sinjiensis*.

Acartia grani is typical of coastal, semi-confined ecosystems, conditioned by a high degree of physical conditions (temperature and salinity) (Calbet and Alcaraz, 1996). Hatching success is responsible for the growth of the culture, being crucial in the reposition of new copepods in the culture systems.

2. Material and methods

2.1 Copepod stock culture

Stock culture was kept according to the conditions described in chapter 1.2.7. Eggs used in this experiment came from our "egg bank". The "egg bank" contains harvested and "cleaned" eggs, which were stored in 1µm filtered and UV-treated seawater in closed falcon tubes at 4ºC.

2.2 Experimental design and setup

For this experiment, eggs from a falcon tube were rinsed in a mesh (55µm) with distilled water to wash out some of the accumulated feces that are stored with the eggs. This procedure is known not to affect viability of unhatched eggs (Knuckey et al., 2005). Eggs were then transferred into a 500ml beaker containing seawater. The beaker was agitated for 15 seconds waiting for a few seconds to allow air bubbles to surface. Then a sample of 1ml was taken from the beaker using an automatic pipette (Pipetman – Gilson 1000uL). This was repeated for five times and the number of eggs in each of these subsamples was counted using a Sedgewick-Rafter counting cell and a microscope (Zeiss – Axioskop 2 Plus). The variability of egg numbers between sub-samples was less than 10%. The average number of eggs in the sub-samples was calculated and used to estimate the concentration of eggs in the stock solution.

Twelve 600ml beakers were incubated with 150 *A. grani* eggs at four different temperatures: 18.0, 22.0, 24.0 and 28.0 ±0.5°C, using water baths; each temperature treatment had 3 replicates. Throughout the experiment, great care was taken to ensure that the correct temperatures were maintained by continuously monitoring the temperature in all water baths and all other conditions were kept very similar in all replicates. Each beaker contained 300ml of seawater (1µm filtered - UV treated), with salinity 38±1 psu and 24 hours light (approximately 1200-1500lux).

2.2.1 Experimental procedure

With the use of a plastic pipette, all hatched nauplii were carefully removed from the beakers and discarded. This procedure was repeated every two hours, until each treatment reached 50% individual hatching time.

At the end of the experiment data were analyzed:

1. The cumulative egg hatch (CUM%) was calculated:

EHR (CUM%) = [(sum of all replicates)*100] / nº total of eggs

2.3 Statistical analysis

The percentage hatching rate data were arcsin transformed prior to analysis. When a significant difference ($p<0.05$) was found, Tukey's multiple comparisons test was used to discern significant differences between treatments. All statistical analyses were conducted using SPSS program, version 20.0. Data are presented as mean ± standard deviation (SD).

3. Results

Figure 1 shows the cumulative egg hatching (CUM%) experiment, for each temperature treatment after a running time of 35 hours.
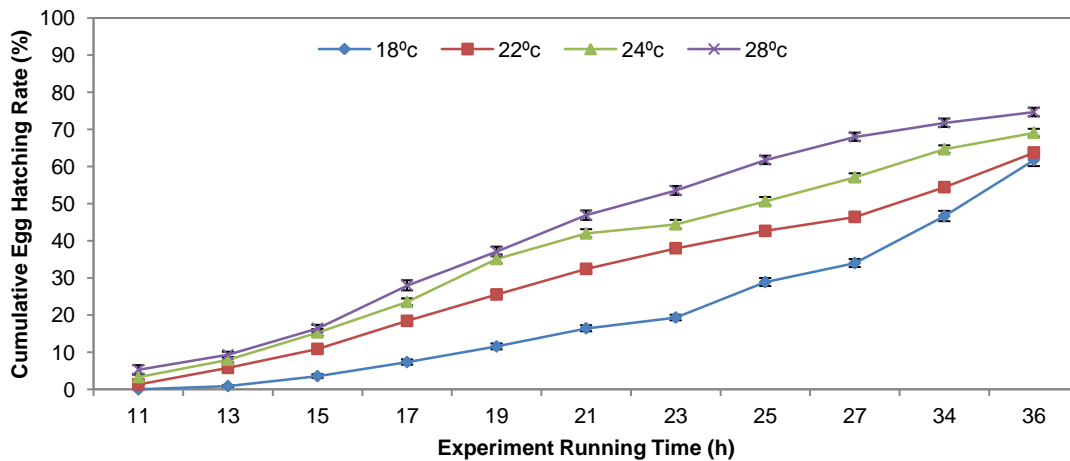
Figure 1 – The 36 hours cumulative egg hatching rate (CUM%) of *Acartia grani* eggs at four different temperatures (18°C, 22°C, 24°C and 28°C). Eggs were incubated under identical conditions of 38±1psu and photoperiod 12L:12D. Data are presented as mean±SD. (One way ANOVA $p=0.576$).

Statistical analysis showed that no significance exists between different temperature treatments ($p>0.05$) at the end of the experiment. First occurrence of *A.grani* nauplii was registered at 11 hours after incubation for temperatures 22ºC, 24ºC and 28ºC. Significant differences were found at 11hours ($p=0.046$), eggs incubated at 28ºC presented the highest number of nauplii (24.0±5.3), followed by eggs incubated at 24ºC with 15.0±2.6 nauplii (Table I). First nauplii occurrence of eggs incubated at 18ºC was registered two hours later, with a maximum number of 4.0±1.2 nauplii.

Acartia grani eggs incubated at 28°C and 24ºC were the first treatments to achieve 50% of hatching rate (29 hours), followed by 22°C and 18°C at 35 hours (Fig. 1).

Table I – The 35h nauplii hatch with four different temperatures (n=450). Data are presented as mean±SD. Different letters indicate significant difference (p<0.05).

Temperatures	Experiment Running Time				
	11h	17h	23h	29h	35h
18ºC	0.0 ± 0.0^a	33.0 ± 3.2^a	87.0 ± 3.4^a	153.0 ± 4.8^a	278.0 ± 7.5^a
22ºC	6.0 ± 0.0^{ab}	83.0 ± 4.3^{ab}	171.0 ± 4.3^{ab}	209.0 ± 4.1^a	287.0 ± 4.3^b
24ºC	15.0 ± 2.6^{ab}	106.0 ± 4.1^b	200.0 ± 5.3^{ab}	257.0 ± 4.7^a	311.0 ± 4.7^{bc}
28ºC	24.0 ± 5.3^b	126.0 ± 6.1^b	241.0 ± 5.4^b	306.0 ± 5.0^a	336.0 ± 5.2^c

4. Discussion

Over the years several studies have been conducted to understand the optimal culture conditions of copepods, due to its importance as live food for aquaculture. Copepod egg development and vitality are known to be affected by temperature (Ban, 1994), and changes in temperature can increase or decrease egg hatch time (Castro-Longoria, 1998). However few studies have regarded hatching rates.

The historical background of the cultivated copepods and therefore also the standard reference temperature for the eggs of *Acartia grani* was 18°C (Calbet and Alcaraz, 1996, 1997; Guerrero and Rodriguez, 1998; Costa and Fernández, 2002). Moreover, the temperature of 18°C seems to be favorable for leaving the dormancy phase (Guerrero and Rodriguez, 1998). However Cunha, (unpublished data, 2010) achieved better production (less mortality, higher hatching rates and development) results at 23°C for the culture of *Acartia grani*. The expectation was that an increase in temperature (≤ 28°C), would improve hatching rates. Guerrero and Rodriguez (1998) showed that *A.grani* eggs hatched when temperatures were higher than 18ºC.

The results of this study showed that a fifty percent egg hatching rate was achieved after a period of 35 hours in all temperatures considered, without significant differences. Nevertheless, as soon as 24 hours of incubation, the 28°C treatment had reached the 50% individual hatching rate (53.6±5.4%), compared to only 19.3±3.4% of hatching success in the 18°C treatment. Analysis of our results seems to indicate that the time necessary to reach 50% egg hatching decreases with increasing temperature. Milione and Zeng (2007) tested hatching rates of *Acartia sinjiensis* (species belonging to the same

family than *Acartia grani*) at 28°C within a 48hours period, and obtained a mean hatching rate approximately of 35%. 35 hours after incubation at 28ºC, *A. grani* eggs used in our study, presented a hatching rate two times higher (74.7±5.2%).

At this point it also interest to mention that within treatments, different levels of activity by the hatched nauplii were observed. Until the first 24 hours, treatments with higher temperatures (24°C and 28°C) presented very active nauplius, which was not verified in the lower temperatures. High temperatures are known to increase the metabolism (Hansen et al., 2010), and the first nauplii that hatch in the high temperatures seem to be looking for food. As time elapsed and the 35 hours of incubation were obtained, copepods were more active in lowest temperature treatments. Unfortunately, since it was not the goal of this study, no further considerations were performed on this subject.

Eggs used in this experiment had 3 months since they were collected and stored. Though considerations could be made if fresh eggs were used, Drillet et al. (2006), showed that *Acartia tonsa* have a hatching rate of 69.9±2.4% with 3 months stored eggs, at 23°C, over 96 hours period. In our case, and as soon as 35 hours after incubation, we reached a cumulative egg hatching rate of 69.1±4.6% at 24°C and 63.8±4.3% at 22°C. Our results prove that use of 3 months eggs of *Acartia grani* still present great potential for culture.

Overall the results found in our study are similar to what other authors have found with *Acartiidae* species. Highest temperatures seem to encourage embryogenesis of the egg, resulting in satisfying egg hatching success, although not significant ($p<0.05$). If lower temperatures are used then more time is needed until the first nauplius appears, since the eggs seem to be in quiescence stage (Hansen et al., 2010).

5. Conclusion

In aquaculture it is fundamental that live food is readily available for use as feed to fish larvae. If we are able to reduce time of egg hatching rate of copepod, reaching at least 50%, it is one of the many factors that will allow us to obtain higher productions in reduced time. Based upon the results of this study, it is recommended that for improving results of the hatching rate, at least within 24 hours after incubation, the temperature must be equal or higher than 24°C.

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The effects of temperature on population growth, egg production and egg hatching success of the calanoid copepod *Acartia grani* (Calanoida: Acartiidae)

1. Introduction

Identifying factors that control the growth of copepods is essential to understanding nutrient and carbon fluxes in the marine environment. There has been considerable debate in the literature about the relative importance of the two main factors that control copepod growth: food and temperature (Ban, 1994). The influence of temperature on growth rates in the wild has been well documented, especially in temperate seas (Middlebrook and Roff, 1986; Davis, 1987; McLaren et al., 1989). Variability in temperature characterizes seasonal succession and directly affects copepod reproduction and development (Ianora et al., 1992). In their review of field measurements of growth rate, Huntley and Lopez (1992) concluded that copepods grow at maximum rates in the field, with an exponential increase in growth rate with temperature over a wide range of habitats. The same authors have also shown that 90% of the growth variability in 33 copepod species can be explained by temperature alone.

Research investigating the effects of temperature and salinity on the productivity of calanoid copepods has shown their clear effects on egg production (Devreker et al., 2009) and egg hatching rates (Hall and Burns, 2002; Peck and Holste, 2006; Holste and Peck, 2006). Most calanoids are broadcasters, shedding eggs singly into the water. The number of eggs spawned in a single event may vary from a few eggs to 50 or more eggs and each spawning event may occur about once every 24 h for extended periods. Free-spawning species such as various *Acartia* species may produce between 11 and 50 eggs female^{-1}day^{-1}, producing a total of up >1200 from one single spawning (Støttrup, 2003).

Rodriguez et al. (1995) on a seasonal scale showed that temperature did not serve to predict completely the egg production rate of *Acartia grani*. Instead, the coincidence of a temperature increase from the winter minima of 13.5 to 18°C appeared to act as synergetic triggers for an explosive reproduction (mean = 79 eggs female^{-1}day^{-1}) of the adult copepods present in the water column. Moreover, nauplii growth and development of various calanoid copepod species (Geiling and Campbell, 1978; Nagaraj, 1992; Takahashi and Ohno, 1996; Payne and Rippingale, 2001; Chinnery and Williams, 2004) have also been described to be temperature related.

Despite, the effects of water temperature on hatching, growth, and fecundity have been well investigated in some *Acartia* species such as *A. clausi* (Landry, 1978), *A. tonsa*

(Heinle, 1969) and *A. calforniensis* (Johnson, 1980), optimum conditions for the development of *A. grani* are not well known.

Evaluating the effects of temperature on overall copepod population dynamics after a certain period of culture rather than focusing on a single stage of the copepod life cycle is likely to provide more complete information for the purposes of aquaculture, as analysis of population growth provides a summary of the effects of environmental factors on the inter-related parameters mentioned above that affect ultimate productivity (Milione and Zeng, 2008). Furthermore, this abiotic parameter is relatively easy to be manipulated and has a strong impact in copepods culture.

The major purpose of this study was to investigate the effects of water temperature on the development of *A. grani* based on rearing experiments in the laboratory, with special attention to the relationship between water temperature and mortality, fecundity and developmental rates of the species.

2. Material and methods

2.1 Microalgae culture

Microalgal culture used for the experiment was *Rhodomonas marina*. For further details concerning algae culture conditions and maintenance protocols, please refer to chapter 1.3.2.

2.2 Copepod stock culture

Stock culture was kept according to the conditions described in chapter 1.2.7. Adults used in this experiment were collected from a parental culture tank with 12 days, since eggs were incubated. They were collected with a 200µm sieve and transferred to the respective treatment.

2.3 Experimental design and setup

Three separate experiments were carried out to assess the effect of temperature in *A.grani* culture productivity, i.e. (1) egg hatching rate, (2) egg production rate and (3) population increase over a 12 day culture period.

Four treatments (4 replicates) were considered on each experiment:
Treatment 1: *A.grani* culture at 18°C
Treatment 2: *A.grani* culture at 22°C

Treatment 3: *A.grani* culture at 24°C

Treatment 4: *A.grani* culture at 28°C

Throughout all the experiments, great care was taken to ensure that the correct temperatures were maintained by continuously monitoring the temperature in all water baths.

Experiments and acclimatization were carried out under similar conditions: seawater 1µm filtered - UV treated; salinity 38±1psu; photoperiod 12L:12D and fed with *Rhodomonas marina* at $1500µgCL^{-1}$, a carbon concentration known to saturate copepod feeding (Kiørboe et al., 1985). Carbon concentrations were calculated according to Strathmann (1967).

2.3.1 Acclimatization to different temperatures

Approximately 200 *A. grani* adults were acclimatized to each temperature for 3 days in 5L aquariums filled with seawater (1µm filtered - UV treated), with salinity 38±1psu and gentle aeration. Each aquarium received $1500µgCL^{-1}$ of microalgae every day. *Rhodomonas marina* concentrations in each aquarium were determined daily using a haemocytometer under a microscope (Zeiss – Axioskop 2 Plus). Temperatures and oxygen were monitored daily with the use of a thermometer and oximeter (Oxyguard-Handy Polaris).

2.3.2 Egg Hatching Rate experiment (EHR)

As in population increase experiment, previously acclimatized copepods were collected into 600ml beakers with a sieve of 200µm. Adult's collection to new beakers was to warranty that the eggs used in the experiment had a maximum of 24 hours. The freshly produced eggs were carefully collected with a 55µm sieve of each treatment and total eggs were counted with *Sedgwick-Rafter* chamber. The eggs were rinsed and put in distilled water for 2 min to kill any possible nauplii. Then 40 to 60 eggs of each treatment were randomly distributed into 100ml beakers, a total of 5 replicate beakers *per* temperature were used.

Egg hatching success was estimated for each treatment by calculating the difference between the number of eggs unhatched and the hatched nauplii, at 10, 20, 24, 30 and 48 hours after incubation. Counting was done with a *Sedgwick-Rafter* chamber, as above.

2.3.3 Egg Production experiment (EP)

Like in the two previous experiments, copepods were subjected to a three day period of acclimatization. With the sieve 200µm, 5 active mature females were collected and carefully transferred to 100ml beakers. There, were 3 replicates *per* temperature and hence a total of 12 beakers. 24 hours later, all the eggs produced by the females were collected with 55µm sieve and count using *Sedgwick-Rafter* cell counter and a microscope (Zeiss – Axioskop 2 Plus).

Following the procedure described above, new females were randomly selected daily from the 5L aquarium culture and transferred into a new set of twelve 100ml beakers containing fresh filtered seawater and microalgae to obtain individual 24h egg output for each of 3 consecutive days. The daily replacement of females ensures that the new females are fertilized and healthy, being ready to the egg production experiment.

2.3.4 Population Growth experiment

After 3 days of acclimatization, 12 healthy (actively swimming and intact appendages) adults (4 Males and 8 Females) were transferred into 600ml beakers filled with 300ml seawater and without aeration. A total of 4 replicate beakers were established for each treatment. Every morning 30% of the culture water was exchanged with a siphon of 55µm mesh to prevent the loose of eggs or nauplii. After water exchanged, a sample (2ml) was collected to determine microalgal concentration on the water column, and readjust if necessary the $1500\mu gC.L^{-1}$. *A.grani* is a suspension feeder, therefore it is important to supply phytoplankton in two meals (one in the morning and the second afternoon), to ensure that microalgae stay more time in the water column.

From previous observations, we knew that a complete life cycle would be possible in 9-12 days. After 12 days, content from each beaker was drained through a 55µm sieve and all eggs, nauplii, copepodites and adults retained were fixed with 4% formaldehyde and stored at 4°C. The counting of *A.grani* samples was made using a *Sedgwick-Rafter* cell counter and a microscope (Zeiss – Axioskop 2 Plus).

Sex ratio was determined based on a sample of all the collected adults (50% of population count).

The specific population growth rate (*K*) of *Acartia grani* was calculated using the following formula (Omori and Ikeda, 1984; Hada and Uye, 1991):

$$K = (ln N_t - ln N_0)/t$$

Here, *t* is the culture days (12) and N_0 and N_t are the initial and final density of copepods, respectively.

In addition, doubling time (D_t) was calculated by dividing $\log_e 2$ by the populations growth rate (K) according to the following formula (James and Al-Khars, 1986):

$$D_t = (\log_e 2)/K$$

2.4 Statistical analysis

Data from all experiments were analyzed using one-way ANOVA. When significant differences ($p<0.05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments ($p<0.05$). All statistical analyses were conducted using SPSS, version 20.0. Data are presented as mean±standard deviation (SD).

3. Results

3.1 Egg Hatching Rate (EHR)

Figure 1 presents the results of the egg hatching rate experiment. Twelve hours after incubation hatching rates were very low in all temperature treatments.
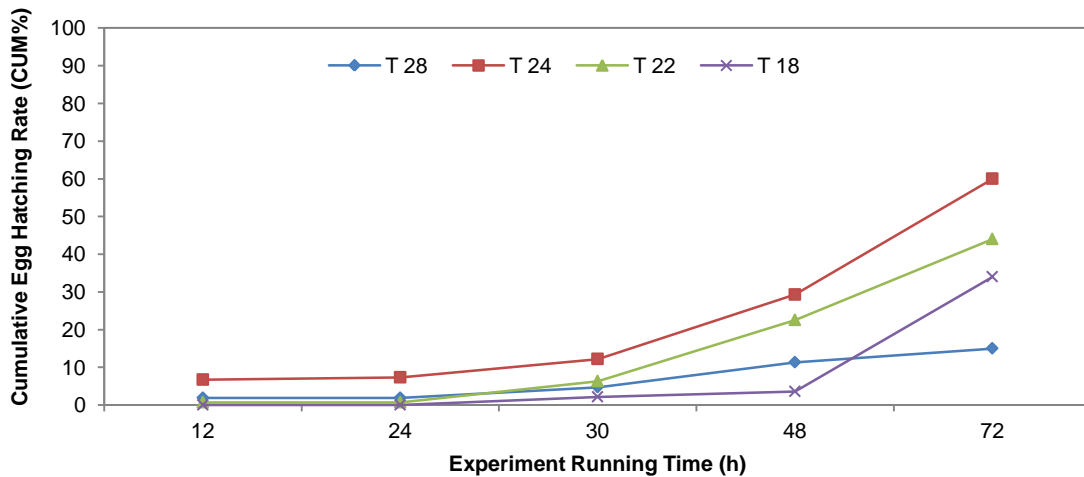
Figure 1 – The cumulative egg hatching rate (CUM%) of *Acartia grani* eggs produced by adults with four different temperatures, over 72 hours. Eggs were incubated under identical condition of 38±1psu and photoperiod 12L:12D.

First eggs incubated at 18ºC hatched only 30 hours later (2%). At the same observation hour, highest mean hatching rate was of 12.2% at 24ºC. Until 48h, EHR at 18ºC was significantly lower than eggs produced at the remaining temperature treatments.

Compared to 48h EHR, 72h hatching rates improved across temperature treatments, except for eggs produced at 28ºC, which increased only to 15% final mean EHR. Egg hatching rate was highest at 24ºC, reaching a mean value of 60.0%, followed by eggs incubated at 22ºC (44%).

3.2 Egg Production (EP)

Results of 24h egg production (EP; eggs female^{-1}day^{-1}) of *Acartia grani* during 4 consecutive days are presented in Figure 2. As different females were used for each experimental day, it is possible to observe that regardless of the temperature treatment there is variation in the mean egg number *per* females *per* day. Still, within days no significant differences were observed between treatments (p>0.05), and both maximum (10.5±1.5) and minimum (1.4±0.6) EP were found on eggs laid at 18ºC on day 4 and day 2, respectively. Since no significant differences were detected within each temperature treatment, data were then pooled to calculate the overall mean 24 EP of the four days (Table I). Though egg production rate was highest in *A. grani* cultured at 18ºC (7.0±2.7), again no significant differences were found on the EP of *Acartia grani* subjected to the four considered temperatures (p=0.76).
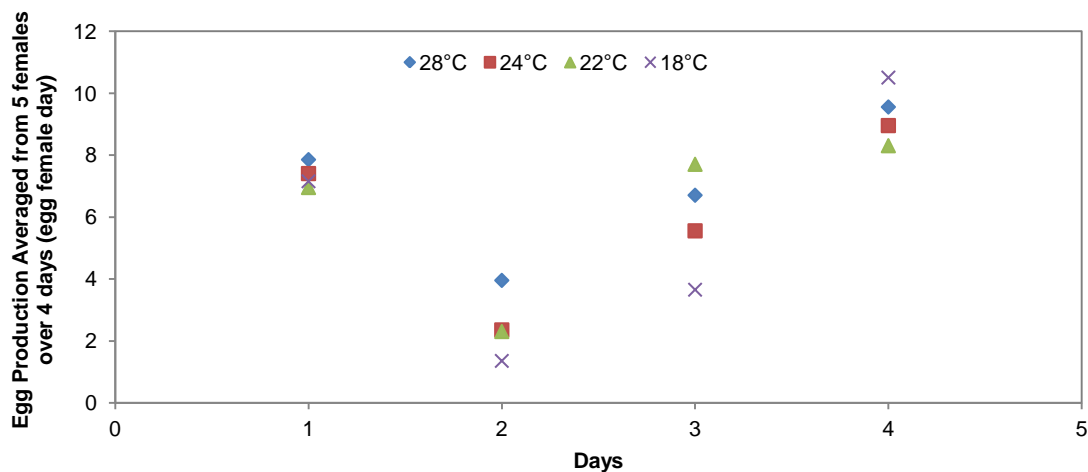
Figure 2 – Mean egg production (eggs female^{-1}day^{-1}) of *Acartia grani* with 4 different temperatures on the 4 experimental days. Data are presented as mean±SD. (Day 1 p=0.86; Day 2 p=0.08; Day 3 p=0.32; Day 4 p=0.99).

Table I – Effects of temperature on averaged 24h egg production of *Acartia grani*. For each treatment, 24h egg production was averaged from 5 females over 4 days. Data are presented as mean±SD. (One way ANOVA *p*=0.76).

Temperature	Eggs female^{-1}day^{-1}
28°C	5.7 ± 4.0[a]
24°C	6.3 ±4.0[a]
22°C	6.1 ±3.6[a]
18°C	7.0 ±2.7[a]

3.3 Population Growth

The results of population growth experiment showed that temperature did not have significant effect (*p*>0.05) on the population growth of *A. grani* (Fig.3) if All Stages were included (All Included-eggs, nauplii, copepodite and adults). Still highest mean final population was observed for *A. grani* cultured at 18°C (663.0±52.2) followed by 28 °C treatment with 640.3±74.7 individuals. On the other hand, if egg stage was not included (All post-egg-stages) highest mean final population number was observed at *A. grani* cultivated at 22°C (216.3±25.0), while lowest population was observed for 24°C temperature treatment (136.5±15.8), again without significance differences.
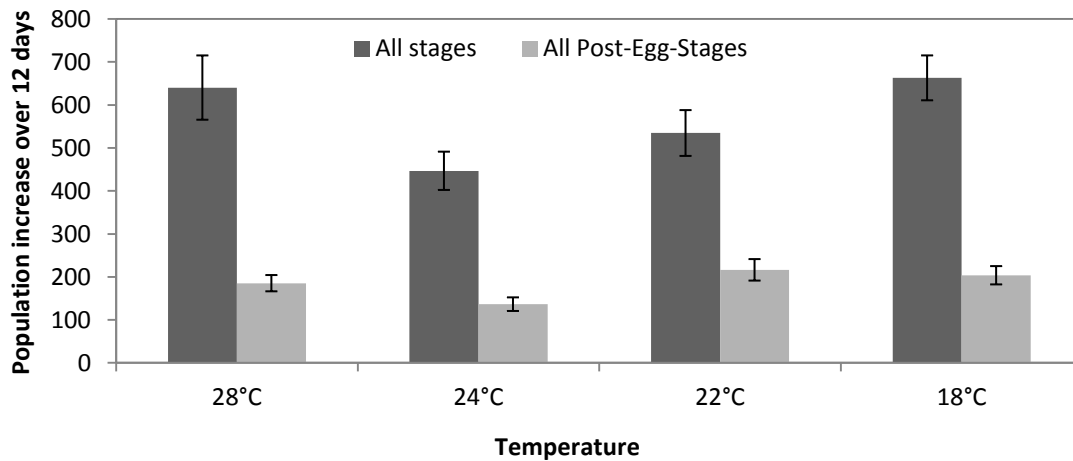
Figure 3- Mean final total population of *Acartia grani* cultured at four different temperatures for a 12 day period. Initial population was 8 females: 4 males Data are represented as mean±SD. All Stages bar: eggs, nauplii, copepodites, adults; All post-egg-stages bar: nauplii, copepodites, adults. Different letters on the tops of bars indicate significant differences (*p*<0.05). One way ANOVA: All stages included *p*=0.86; All Post-eggs-stages *p*=0.82.

Within the population, except for the adult stage, there were no notable differences in the distribution of the various life-stages, i.e. eggs, nauplii, copepodites of *A.grani* cultured

at different temperatures. Adult population was significantly higher (p=0.025) at 28ºC than mean adult population cultured at 18 ºC (Table II).

Table II - Mean number of four life stages (eggs, nauplii, copepodites and adults) within the population of *Acartia grani* cultured for 12 days at four different temperatures (18ºC, 22ºC, 24ºC, 28ºC) from an initial number of 12 adults. (K) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences (p<0.05). Data are represented as mean±SD.

Temperature	Eggs	Nauplii	Copepodites	Adults	K	D_t
28°C	455.0 ±299.9[a]	10.3 ± 4.7[a]	118.3 ±56.9[a]	56.7 ± 28.9[a]	0.32 ±0.04[a]	2.16 ±0.30[a]
24°C	310.3 ±271.8[a]	37.5 ±30.2[a]	82.5 ±102.1[a]	16.5 ±19.0[ab]	0.28 ±0.07[a]	2.84 ±0.39[a]
22°C	318.3 ±229.4[a]	97.7 ±66.0[a]	98.3 ±96.0[a]	20.3 ± 17.6[ab]	0.30 ±0.06[a]	2.35 ±0.47[a]
18°C	459.3 ±196.0[a]	155.3 ±99.3[a]	40.8 ± 27.4[a]	7.8 ± 1.0[b]	0.33 ±0.02[a]	2.07 ±0.14[a]
	p= 0.764	p= 0.051	p= 0.595	p= 0.031	p=0.489	p= 0.374

The specific population growth rate (K) of *A. grani* was calculated for all treatments (Table II). It ranged from 0.28 to 0.33, the lowest at 24°C and highest both at 18°C and 28°C. Population doubling time (D_t) showed that 24°C was the treatment that needed more time to grow (2.84) and that the 18°C treatment presented the lowest value (2.07). For all treatments no significance differences where found in K and D_t parameters.

Sex ratio of *A. grani* after a 12 day culture period was similar (p>0.05) between different temperatures, except for adults cultured at 18ºC that presented a skewed tendency sex ratio toward males (80%F:20%M) (Fig. 4).
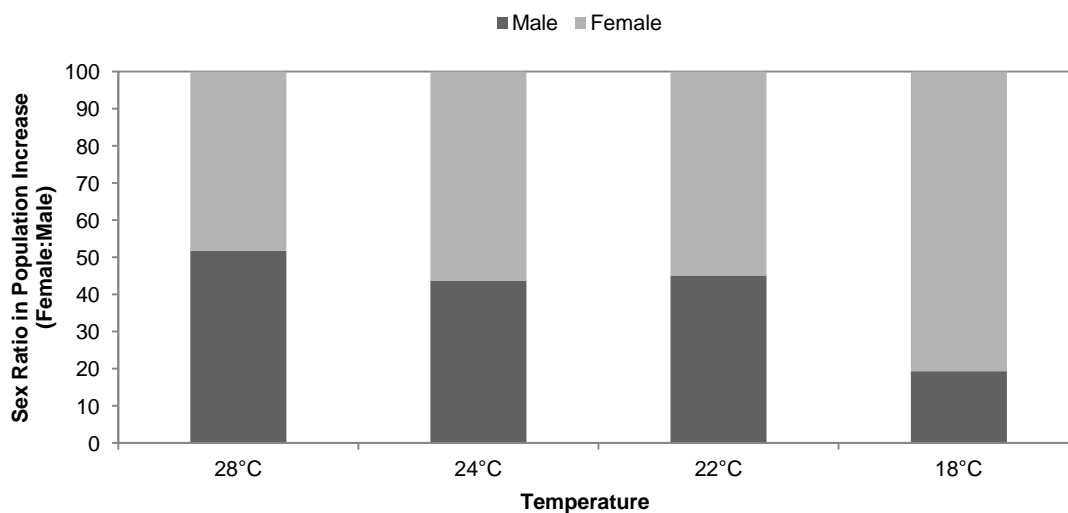
Figure 4 – Sex ratio of *A.grani* in the population increase experiment after 12 days.

Mortality of the initial adult population was registered and results revealed that on the first three experimental days, between 10% (18ºC) and 21% (22ºC) of the initial adults had died. By the 8th experimental day, mortality reached 55.6% at 18ºC, followed by 22ºC with a mean mortality rate of 54.0%. From that day onwards mortality was not counted, due to possible misleading of initial incubated adults with born grown adults during the experiment.

4. Discussion

Temperature can be relatively easily manipulated in aquaculture hatcheries, where mass culture of copepods is desirable as a means of providing live prey for culture animals (Milione and Zeng, 2007).

The aim of this study was to evaluate the importance of temperature on the egg hatching rate, egg production and population increase. Aside from egg production, copepod productivity may also be linked to other factors, such as the hatching rate of eggs and subsequent survival and development of the nauplii and copepodites, as well as life expectancy and sex ratio of the adults (Knuckey et al., 2005). Egg production and hatching rate are normally lower at low temperatures (Ambler, 1985; Uriarte et al., 1998), and generally increase with increasing temperature up to a thermal threshold, after which decline begins. This was reported by Takahashi and Ohno (1996) in *A. tsuensis*; White and Roman (1992), Holste and Peck (2006) reported it for *A. tonsa* and Milione and Zeng (2008) for *A. sinjiensis.*

Commonly *A. grani* was cultured at 18°C (Calbet and Alcaraz 1996, 1997; Guerrero and Rodriguez, 1998; Costa and Fernández, 2002). However, new data (Cunha, 2010 unpublished data) suggest that better production could be achieved with warmer temperatures. Leandro et al. (2006) found that *Acartia clausi*, in special the northern population, had a faster growth rate when reared at high temperatures. Also, Chinnery and Williams (2003) concluded that highest temperatures influenced survival of nauplii of *Acartia* species (*A. bifilosa, A. clausi, A. discaudata* and *A. tonsa*). Takahashi and Ohno, (1996) studying *A. tsuensis* showed that high hatching success, normal isochronal development, minimum mortality and high fecundity was obtained consistently at around 25.0°C, when compared with temperatures that ranged between 17.5°C and 30 °C.

In our study, some of the data do not concur with the above mentioned studies. EHR increased with increasing temperature, though EHR in the 28°C treatment had the lowest rate after 72 hours of incubation. Contrary to our findings, Rodriguez et al. (1995) observed in August (22°C to 26°C) with *A.grani* that 89% of eggs produced hatched in 48-

72 hours. In the previous study that we realized with *Acartia grani* incubated at different temperatures (Chapter I), this same temperature showed the highest egg hatching rate, being included in the optimal range of temperatures for induction of better EHR of *A.grani*. We believe that low egg hatching rate at 28°C was due to either the appearance of bacteria in the culture medium or bad quality of the eggs. According to Peck and Holste (2006) it is possible that using eggs produced from different cohorts (or even from different days from the same cohort) could have contributed to variability in egg hatch success. Moreover, on our first experiment hatching rates were found as soon as eleven hours after incubation. On the current experiment, first hatch occurred further later (thrity hours) and within the temperature treatment of 18ºC.

Egg production data revealed that averaged 24h EP was similar in *A. grani* cultured at different evalueted temperatures. Koski and Kuosa (1999) found that for 24ºC affected negatively survival and egg production of A. *bifilosa*. Similarly, *Acartia tonsa* egg production appears to be inhibited when temperature exceeds 27 ºC (White and Roman, 1992). However, in our experiment, on the first two days, the highest temperature (28°C) presented the maximum egg production, although no significances were found. Holste and Peck (2006) found for *Acartia tonsa* an increase in egg production rate with increasing temperature that was far stronger than that estimated from studies of other calanoid copepod species.

Temperature also has a direct influence on the development rate and the survival of copepod nauplii (Mauchline, 1998; Dussart and Defaye, 2001; Peterson, 2001). In our study, population growth of *Acartia grani* was also not significantly affected by temperature. The highest mean final population was observed at 18ºC if all stages were considered, but the 28 ºC treatment had only less 23 individuals. Taking into account that the same cohort of copepods was used and analyzing egg hatching rate and egg production at 18 ºC, results of final population growth if all stages are included could be easily explained at this temperature. However, due to possible lower metabolic rates, adult population was only 7.8±1.0 individuals with significant differences to 56.7±28.9 adults of the 28 ºC treatment. Development time decreased as the temperature increased.

According to Chinnery and Williams (2004), temperature is considered the most important fact affecting juvenile development and growth rate in *Acartia* congeners. The development time of P. *elongata* copepodites (Ozaki and Ikeda 1997) and S. *tenellus* nauplii (Kimoto et al. 1986) also decreased with increasing temperature. Landry (1975) explained this effect by assuming that development is controlled by a series of biochemical reactions, the rates of which are regulated by temperature. Moreover, if similar results to our previous egg hatching rate experience had been found (EHR at 28ºC was of 74.3±1.2%), the number of eggs and nauplii would have certainly increased.

5. Conclusion

This study proves that *Acartia grani* had the ability to adapt to different culturing temperatures, and developed successfully. Calbet and Alcaraz (1996, 1997); Guerrero and Rodriguez (1998); Costa and Fernández (2002) all worked with *Acartia grani* at lowest temperatures (18 ºC) and obtained good results. However when production is needed in aquaculture facilities, the optimal and faster way is required, and Cunha, unpublished data, (2010) showed that higher (23 ºC) temperatures had better results.

Results of this study allow us to conclude that the optimal temperature for *A.grani* production ranges between 24 ºC and 26 ºC.

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The effects of photoperiod on population growth, egg production and egg hatching success of the calanoid copepod *Acartia grani* (Calanoida: Acartiidae)

1. Introduction

The productivity of copepod culture is affected by a range of factors, including diet, stocking density and environmental conditions for culture, such as temperature, salinity and photoperiod (Ambler, 1986; Rodriguez et al., 1995; Klepper et al., 1998; Koski and Kuosa, 1999; Castro-Longoria, 2003; Leandro et al., 2006). Photoperiod is one of the most significant cues for seasonality in nature (Hairston and Kearns, 1995) and could therefore be a key factor controlling female copepod reproductive status and population dynamics. To evaluate the productivity of a copepod culture we firstly need to link the rate of eggs produced by the females. The results of a number of previous studies suggest that light regime can markedly influence diel rates of egg production in *Acartia tonsa* (Stearns et al., 1989; Cervetto et al., 1993; Peck and Holste, 2006). However, aside from egg production, other biological parameters, such as subsequent egg hatching success (Jepsen et al., 2007), nauplii and copepodite development rates (McKinnon et al., 2003), sex ratio and life expectancy of adults could also significantly affect copepod productivity in culture (Milione and Zeng, 2007). Hence, a systematic evaluation of the effects of photoperiod on all these parameters is likely to provide comprehensive information helping to improve productivity of copepod culture.

Despite photoperiod is an environmental parameter that can be easily manipulated with minimum costs in aquaculture hatcheries (Chinnery and Williams, 2003), light levels and periodicity are rarely reported in the literature. In many cases no particular light is provided with low light levels being applied. In nature, high solar radiation is harmful to copepods; hence, adults show negative phototaxis during the day and positive phototaxis during the night (Dussart and Defaye 2001), and several calanoids are known to spawn at night (Mauchline, 1998).

In order to maximize the productivity of intensive culture for the use as live feed in aquaculture, experiments were conducted to investigate the effects of photoperiod on egg production, hatching success and development rate of *Acartia grani*.

2. Material and methods

2.1 Microalgae culture

Microalgal culture used for the experiment was *Rhodomonas marina*. For further details concerning algae culture conditions and maintenance protocols, please refer to chapter 1.3.2.

2.2 Copepod stock culture

Stock culture was kept according to the conditions described in chapter 1.2.7. Adults used in this experiment were collected from a parental culture tank with ±12 days, since eggs were incubated. They were collected with a 200µm sieve and transferred to the respective treatment.

2.3 Experimental design and setup

Three separate experiments were conducted to investigate the effects of photoperiod on major parameters related to *A.grani* culture productivity, i.e. (1) egg hatching rate, (2) egg production rate and (3) population increase over a 12 day culture period.

Five photoperiod conditions of Light: Dark conditions were setup for all the experiments with 4 replicates each:

Treatment 1: *A.grani* culture with 24L:0D
Treatment 2: *A.grani* culture with 18L:6D
Treatment 3: *A.grani* culture with 12L:12D
Treatment 4: *A.grani* culture with 8L:18D
Treatment 5: *A.grani* culture with 0L:24D

The light intensity in all treatments was kept constant at about 1000-1500 lux, meansured with Lux meter (Testo 540-Lux). All experiments and acclimatization were carried out under similar conditions: seawater 1µm filtered - UV treated; salinity 38±1psu; 24±0.5 °C.

2.3.1 Acclimatization to Photoperiod conditions

Approximately 200 *A. grani* adults, collected from a stock culture tank, were acclimatized to each photoperiod for 3 days in 5L aquarium with seawater and gentle aeration. Copepods were fed daily (morning and afternoon) $1500\mu gCL^{-1}$ (±31600cel.ml^{-1})

of *Rhodomonas marina*. Microalgal concentrations were determined daily using a haemocytometer (*Malassez* counting chamber) under a microscope (Zeiss – Axioskop 2 - plus).

2.3.2 Egg Hatching Rate experiment (EHR)

Acclimatization of *A. grani* adults for the hatching rate experiment followed the same procedure as per population growth experiment. Adults were then collected with a 200µm sieve to new beakers to warrant that the eggs used in the experiment were fresh. After a 24h period, the freshly produced eggs of each photoperiod treatment were carefully collected with a 55µm sieve and total number of eggs was counted with a *Sedgwick-Rafter* chamber. Samples of 40 to 60 eggs (counted under a microscope) were randomly distributed in each of the five replicates *per* treatment.

Egg hatching success was estimated for each photoperiod by calculating the difference between the number of eggs unhatched and the hatched nauplii at 10, 20, 24, 30 and 48 hours after incubation.

2.3.3 Egg Production experiment (EP)

With the 200µm sieve adults were collected from the acclimatization 5L aquarium and placed in a Petri dish with filtered seawater. Seventy five mature females were carefully transferred to fifteen 100ml beakers (five females per replicate; three replicates per treatment). Each replicate contained fresh filtered seawater and *Rhodomonas marina*.

After 24 hours freshly produced eggs were collected with 55µm sieve and counted under a microscope (Zeiss – Axioskop 2 - plus) with a cell counter *Sedgwick-Rafter*.

For three consecutive days, the same procedure was followed and new females were randomly selected from the acclimatization aquarium and transferred into a new set of twelve 100ml beakers. The daily replacement of females ensures that the new females are fertilized and healthy, being ready to the egg production experiment.

2.3.4 Population Growth experiment

Adult *A. grani* were siphoned from the acclimatization aquarium onto a 200µm sieve and were placed in a Petri dish with a small amount of seawater. Individuals were randomly captured using a fine-tipped pipette. Twelve healthy (actively swimming and

intact appendages) adults (4 Males and 8 Females), were transferred into 600ml beakers filled with 300ml seawater and without aeration. A total of 20 replicate beakers were established with 4 replicates for each treatment. Every morning 30% of the culture water was exchanged with siphon 55μm mesh to prevent the loose of eggs or nauplii. After water exchanged, a sample was collected to determined microalgal concentrations on the water column, and readjust if necessary the 1500μgC.L^{-1}, a carbon concentration known to saturate copepod feeding (Kiørboe *et al.* 1985). Carbon concentrations were calculated according to Strathmann (1967). *A.grani* is a suspension feeder, therefore it is important to supply phytoplankton in two meals (one in the morning and the second at afternoon), to ensure that microalgae stay more time in water column.

The population growth experiment lasted 12 days, after which all the contents of each replicate beaker was drained onto a 55μm sieve mesh and all eggs, nauplii, copepodites and adults retained were fixed with 4% formaldehyde and stored 4°C until further counting. The counting of *A.grani* samples were made using a *Sedgwick-Rafter* cell counter and a microscope (Zeiss – Axioskop 2 - plus).

The specific population growth rate (*K*) of *Acartia grani* was calculated using the following formula (Omori and Ikeda, 1984; Hada and Uye, 1991):

$$K = (ln N_t - ln N_0)/t$$

Here, *t* is the culture days (12) and N_0 and N_t are the initial and final density of copepods, respectively.

In addition, doubling time (D_t) was calculated by dividing $log_e 2$ by the populations growth rate (*K*) according to the following formula (James and Al-Khars, 1986):

$$D_t = (log_e 2)/K$$

2.4 Statistical analysis

Data from all experiments were analyzed using one way ANOVA. When significant differences ($p < 0.05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments ($p < 0.05$). All statistical analyses were conducted using SPSS, version 20.0. Data are presented as mean±standard deviation (SD).

3. Results

3.1. Egg Hatching Rate (EHR)

Figure I shows the results of the egg hatching rate experiment. No hatching occurred in the first two observation hours (12 and 24 hours after incubation) in all photoperiod conditions. For that reason, these results were excluded from statistical analysis. At 30h, *A. grani* eggs subjected to photoperiods of 24L:0D and 6L:18D, presented an EHR of 4.3% and 3.5% respectively. Two days after incubation, hatching had occurred in all photoperiods considered. Still, EHR was very low, not reaching 50%.The highest rate was observed for 6L:18D treatment (21.0%), being the lowest within the photoperiod of 12L:12D (2.8%).
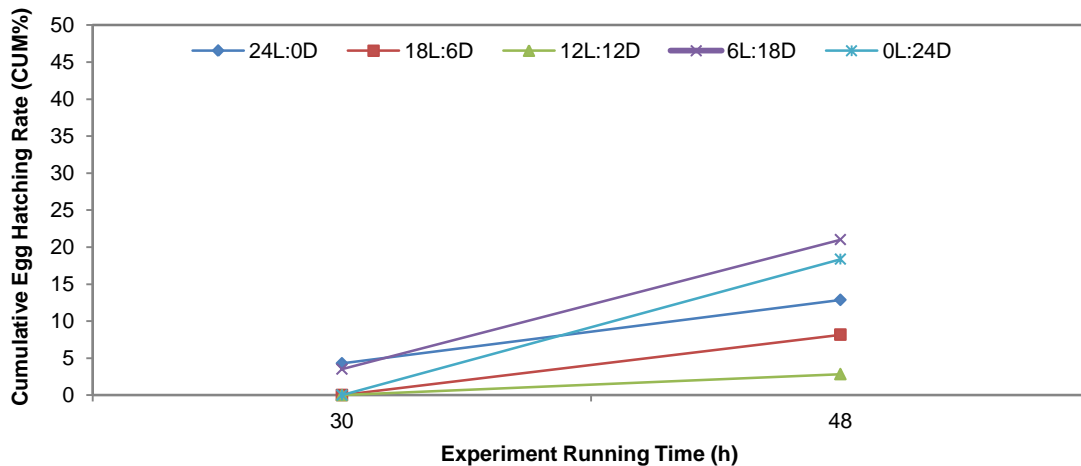
Figure 1 – The cumulative egg hatching rate (CUM%) of *Acartia grani* eggs produced by adults at five different photoperiod over 48 hours. Eggs were incubated under identical conditions of 24±0.5°C and 38±1psu.

3.2 Egg Production (EP)

Under different photoperiod regimes, the daily egg production rates *per* female of *A.grani* over 3 consecutive days are showed in Figure 2. No clear trend was observed between different photoperiod treatments ($p>0.05$) *per* day. Oscillation of egg production between treatments and days was evident. Adults subjected to a photoperiod 12L:12D, presented the highest and lowest value of egg production: 9.5±1.9 (day 1) and 1.3±0.8

(day 3), respectively. Second highest EP was registered with the photoperiod of 0L:24D (8.7±4,6 eggs.female^{-1}day^{-1}) in day one.
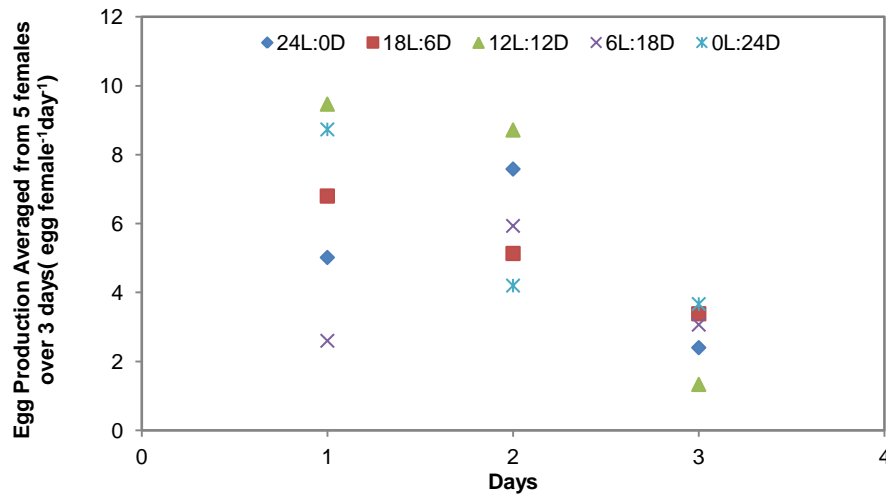
Figure 2 – Mean egg production (eggs female^{-1}day^{-1}) of *Acartia grani*, with 5 different photoperiods on the 3 experimental days. Data are presented as mean±SD. (Day 1 *p*=0.54; Day 2 *p*=0.63; Day 3 *p*=0.68).

Since no significant differences were detected within each photoperiod treatments, data were then pooled to calculate the overall mean 24 EP of the three days (Table I). Though egg production was highest in A. *grani* cultured at 12L:12D (6.5±4.6 eggs female^{-1}day^{-1}), again no significant differences (*p*=0.62) were found on the EP of *Acartia grani* subjected to the five considered photoperiods.

Table I – Effects of photoperiod on averaged 24h egg production of *Acartia grani*. For each treatment, 24h egg production was averaged from 5 females over 3 days. Data are presented as mean±SD (One way ANOVA *p*=0.62).

Photoperiod	Eggs female^{-1}day^{-1}
24L:0D	5.0 ±2.7[a]
18L:6D	5.1 ±2.3[a]
12L:12D	6.5 ±4.6[a]
6L:18D	3.9 ±1.8[a]
0L:24D	5.5 ±3.8[a]

3.3 Population Growth

Figure 3 presents the results of population growth experiment after 12 days of culture at different photoperiods. The results showed that photoperiod did not have a significant effect on the population growth of *Acartia grani* ($p>0.05$). The average final population numbers are presented in two categories: All stages included (i.e. eggs, nauplii, copepodites, adults); and All post-eggs-stages (i.e. excluding eggs). The highest final population All stages included was found in treatment 12L:12D, reaching a mean population of 964.0±410.7 individuals, from 12 initial adults. Similar results were found under no light conditions (0L:24D) (951.0±310.4). At constant light, population was reduced to 658.0±187.1 individuals, though not statistically different. The results of population growth when all post-eggs-stages were considered significantly reduced the number of individuals in all photoperiod treatments. Moreover, highest number was now found under the photoperiod of 0L:24D, with a population of 253.3±58.4 individuals, while lowest population was observed at 18L:6D (98.5±15.2). Still, no significant differences were observed between photoperiod treatments.
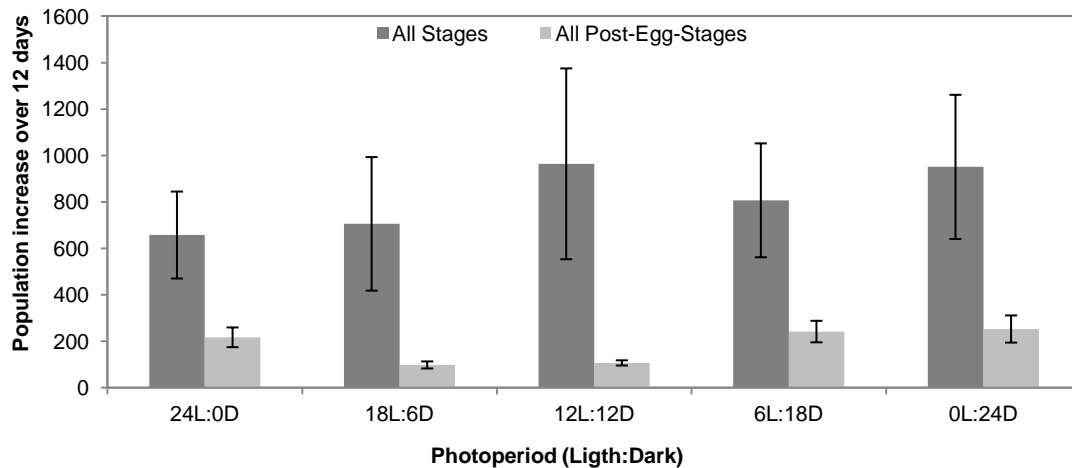
Figure 3- Mean final total population of *Acartia grani* cultured at five different photoperiods for a 12 day period. Initial population was 8 females: 4 males. Data are represented as mean±SD. All Stages bar (all life stages: eggs, nauplii, copepodites, adults); All post-egg-stages bar (nauplii, copepodites, adults). Different letters on the tops of bars indicate significant differences ($p<0.05$). One way ANOVA: All Stages included $p=0.94$; All Post-Eggs-Stages $p=0.07$; (24Light: 0Dark; 18Light: 6Dark; 12Light: 12Dark; 6Light: 18Dark; 0Light: 24Dark).

Within the population, there were also no significant differences ($p>0.05$) in the distribution of the various life-stages, i.e. eggs, nauplii, copepodites and adults of *A.grani*

cultured at different photoperiods (Table II). The specific population growth rate (K) of *A. grani* was calculated for all treatments (Table II). It ranged from 0.32 to 0.35, the lowest was in 24L:0D and 18L:6D treatments and highest was observed in 12L:12D and 0L:24D. Population doubling time (D_t) (Table II) showed that 24L:0D was the treatment that needed more time to grow (2.18±0.36). For all treatments no significance differences where found in K and D_t parameters.

Table II - Mean number of four life stages (eggs, nauplii, copepodites and adults) within the population of *Acartia grani* cultured for 12 days at five different photoperiods from an initial number of 12 adults. (K) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences ($p<0.05$). Data are represented as mean ±SD.

Photoperiod	Eggs	Nauplii	Copepodites	Adults	K	D_t
24L:0D	440.3 ±197.4[a]	107.0 ± 93.3[a]	85.5 ±58.9[a]	25.0 ±13.5[a]	0.32 ±0.05[a]	2.18 ±0.36[a]
18L:6D	607.3 ±248.0[a]	49.5 ±45.2[a]	29.2 ±43.7[a]	19.8 ±12.6[a]	0.32 ±0.04[a]	2.09 ±0.26[a]
12L:12D	857.0 ±534.4[a]	39.5 ±15.4[a]	44.5 ±31.4[a]	23.3 ±13.6[a]	0.35 ±0.06[a]	2.03 ±0.44[a]
6L:18D	565.0 ±450.2[a]	86.5 ±76.6[a]	124.0 ±96.8[a]	31.8 ±17.3[a]	0.33 ±0.05[a]	2.07 ±0.26[a]
0L:24D	697.8 ±344.4[a]	115.8 ±71.2[a]	120.5 ±50.0[a]	17.0 ±6.3[a]	0.35 ±0.04[a]	1.97 ±0.26[a]
	$p= 0.614$	$p= 0.413$	$p= 0.140$	$p= 0.582$	$p= 0.886$	$p= 0.913$

Sex ratio of *A. grani* after a 12 day culture period was similar ($p>0.05$) between different photoperiods (Fig.4). Highest incidence of females was registered in 24L:0D treatment (57.9%) followed by 0L:24D (57.4%) and 18L:6D (55.7%). Photoperiods of 12L:12D and 6L:18D presented the highest percentage of males.
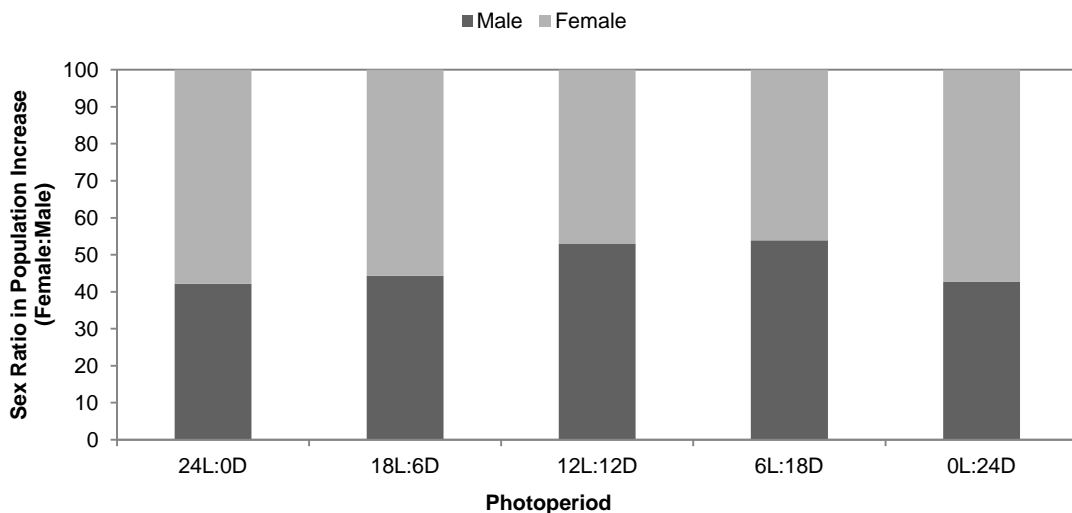
Figure 4 – Sex ratio of *A.grani* in the population increase experiment after 12 days.

Mortality of the initial adult population was registered and results revealed that on the first three experimental days, 4.2% of initial adults died in all treatments with the exception

of 24 light regime that had highest mortality percentage of 6.3%. By the 8th experimental day, mortality reached 14.6% at 12L:12D, followed by 6L:18D with a mean mortality rate of 12.5%. From that day onwards mortality was not counted, due to possible misleading of initial incubated adults with born grown adults during the experiment.

4. Discussion

Over the years copepods are more promising in the aquaculture because of the potential to use as live feed. Interest in large-scale culturing copepods is therefore growing and recent reviews (Støttrup, 2003; Lee et al., 2005; Drillet et al., 2011) discuss culturing techniques and the application of copepods as live prey in marine fish aquaculture. Although photoperiod is a major environmental parameter that can be easily manipulated with minimum costs in aquaculture hatcheries (Chinnery and Williams, 2003), effects of photoperiod on copepod productivity have not been well examined (Peck and Holste, 2006).

In the present study, we analyzed the photoperiod impact on the egg hatching, egg production and population increase of *Acartia grani*. In the egg hatching rate our data showed that, despite no significant differences were observed, *Acartia grani* eggs prefer to hatch in conditions with less light, since better results were obtained within the photoperiod of only 6 hours of light (21.0%). However the results are not satisfactory, when compared with other studies, thus egg hatching rate seemed to be very low in all photoperiod conditions. Moreover, and contrary to our findings, Camus and Zeng (2008), using *A. sinjiensis* obtained the lowest egg hatching rate (72.9±2.6%) at constant darkness (0L:24D) and the highest EHR (87.2±1.4%) at constant light (24L:0D), over 48hours. In 2006, Peck and Holste showed that egg hatching rates for *Acartia tonsa* was highest (85%) in the treatment exposed to more light hours.

Results in egg production demonstrated that photoperiod did not affect egg production *per* female *per* day neither production *per* female over 3 days. Peck and Holste (2006) also reported to *A. tonsa* that total number of egg output was unaffected by photoperiod. Overall the highest averaged 24 hours egg production was obtained in the 12 hour light treatment, taking into account that in day 3, this same light period had the lowest EP. In the treatment without light, the averaged 24 hours egg production was the second highest. In agreement, Stearns et al. (1989) and Peck and Holste (2006) reported that for *Acartia tonsa*, hourly egg production in darkness tended to be more than twice than the hourly rate during light periods. Calbet and Alcaraz (1996) concluded that *Acartia grani* feeding on high food during the night produce more eggs than copepods fed on high

food during the day. Contrasting to our results, Camus and Zeng (2008) working with *Acartia sinjiensis* found with a similar protocol highest egg production in treatments with ≥18h illumination. Different results probably reflect species-specific responses to photoperiod. Some species of *Acartia* and several *Calanus* are known to spawn at night (Støttrup, 2003) but some species have no rhythm. According to Rodriguez et al. (1995), *Acartia grani* would produce eggs continuously, a few at time and not in distinct clutches (Rodriguez et al., 1995).

Population increase over 12 days showed that photoperiod did not have a significant effect on the population growth of *Acartia grani* ($p>0.05$). Again, the 12L:12D photoperiod had the highest results when all stages are included (964±410.7). Within the population, there were also no significant differences in the distribution of the various life-stages. The 24L:0D had the lowest production of eggs in the population growth. Camus and Zeng (2008) suggested that under constant illumination, copepods were probably active 24 h around, which required higher metabolic rate to sustain. Such a high metabolic rate plus high daily egg production probably had depleted their energy reserves, leading to the decrease in egg production. This suggests that high metabolic rate probably influence the mortality percentage in the first 3 days (6.3%) and had influence too in the population growth, shortening the life expectancy.

Sex ratio of *A. grani* after a 12 day culture period was similar ($p>0.05$) between different photoperiods. In 3 treatments (24L:0D, 18L:6D; 0L:24D) higher percentage of females was registered, and the results are in accordance with previous reports for other *Acartia* species (Fleminger, 1985; Medina and Barata, 2004; Camus and Zeng, 2008).

5. Conclusion

In conclusion, based upon the results of this study, photoperiod does not exert a significant effect on the culture of *Acartia grani*. Still, this species seems to present good culture conditions with a photoperiod of 12 hours of light or less. With exception of the egg hatching rate (which was severely low in all treatments), the 12L:12D treatment presented mean better results of culture in order to maximize its productivity. This same photoperiod was suggested for Calanoid species (Støttrup, 2003) as being the most favorable regimen.

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Egg production, egg hatching success and population increase of the calanoid copepod, _Acartia grani_ (Calanoida: Acartiidae), fed different microalgal diets

1. Introduction

Food quality and quantity are probably the most important factors regulating the productivity of copepod culture. As such, the effects of different microalgal diets on egg production (Calbet and Alcaraz, 1996; Kleppel et al., 1998; Koski and Kuosa, 1999; Payne and Rippingale, 2000; Turner et al., 2001), egg hatching success (Shin et al., 2003; Milione and Zeng, 2007), mortality (Kang and Poulet, 2000; Lincoln et al., 2001) and development (Knuckey et al., 2005; Leandro et al., 2006) have been documented for several calanoid species. Most calanoid copepods are filter-feeders, but there is little information on their natural food preference and on the individual food value of the species present in natural phytoplankton. For this reason, the identification of microalgal diets that allow good survival, fast rates of somatic growth and of reproduction, as well as high copepod yields is of paramount importance for larval fish culture (Kleppel et al., 2005; Lee et al., 2006).

There is a wide range of microalgae available for aquaculture, but selection of the algal diet must be based not only for their specific nutritional qualities, but labor requirements and costs involved in algal culture are also important considerations. For large scale and commercial copepod cultivation the diet needs to be as simple as possible, and use commonly grown and robust algal species (Knuckey et al., 2005; Lee et al., 2006).

Among microalgae species commonly used as a diet for the culture of copepods, _Rhodomonas sp._ is often reported as being effective as a sole diet or as part of a mixed algal diet for _Acartia_ species (Støttrup and Jensen, 1990; McKinnon et al., 2003; Knuckey et al., 2005; Morehead et al., 2005; Holste and Peck, 2006; Peck and Holste, 2006). However, reliance upon _Rhodomonas_ can be problematic because it is relatively unstable in culture and is prone to suddenly dying off (Knuckey et al., 2005).

Copepods from the genus _Acartia_ play an important role in the food webs of estuaries in both tropical and subtropical areas (Björnberg, 1981; Mauchline et al., 1998). Generally, they show the higher biomass values in most shallow enclosed bays and estuaries (Azaiteiro et al., 2005; Leandro et al., 2007). This fact may be related to their omnivorous feeding behavior, being able to survive and reproduce under different diets (Kleppel,

1992; Saiz et al., 2007). Such characteristic makes *Acartia* species relatively easy to cultivate at small and large-scale under laboratory conditions, providing enough biomass for use as an alternative live food in marine aquaculture (Støttrup and Nosker, 1997; McKinnon et al., 2003).

Being relatively easy to cultivate *Acartia* species, we cannot forget that in culture systems where the culture medium is not exchanged daily, waste products and superfluous feed may accumulate and generate problems with ciliates and other contaminants, which may cause the culture to collapse.

To date, there have been no studies, on how diet composition may affect the population growth, egg production and egg hatching success of *Acartia grani*. Several authors have suggested that monospecific diets may cause nutritional deficiencies, because of the inadequate content of one or more essential nutrients. To reduce this risk, it has been suggested the use of mixed diets, because their combined nutrient contents are more likely to meet the nutritional requirements of the target species (Brown et al., 1989; Smith et al., 1992). In the case of copepods, this was confirmed by Milione and Zeng (2007), who found that the development rate of the calanoid *Acartia sinjiensis* was significantly better with a mixture of two microalgae than with monospecific diets. Moreover, the different combination of microalgae seems to be a good option to warranty the optimal growth of the different phases of copepods, because different cell sizes can be utilized by the feeding nauplii, copepodite and adult stages.

This research was conducted to investigate the effects of different algal diets on the population growth, egg hatching success and egg production on the population of *A.grani*. The main objective is to optimize their algal diet that would maximize the potential of culture.

2. Material and methods

2.1 Microalgae culture

All of the microalgae utilized in present experiments are common used algal species in aquaculture, therefore relatively easy to culture, with the exception of *Rhodomonas marina*. Three algal species were used in this study: *Rhodomonas marina* (Rho); Tahitian strain *Isochrysis sp*. (T-Iso); *Tetraselmis suecica* (Tet).

R. marina was cultured according to the conditions and maintenance protocols described in chapter 1.3.2, Tahitian strain of *Isochrysis* sp. (T-Iso); *Tetraselmis suecica* (Tet) were inoculated by starter cultures supplied by IPIMAR (Olhão) and were grown by batch method with Nutribloom medium (Annex, Fig.1), at 20±1ºC salinity 25psu, 24h light

in 1L carboys, with continuous aeration. Seawater was 1µm filtered and UV irradiated. The algal cultures were in their exponential growth phase when were used for feeding copepods.

2.2 Copepod stock culture

Stock culture was kept according to the conditions described in chapter 1.2.7. Adults used in this experiment were collected from a parental culture tank with ±12 days, since eggs were incubated. They were collected with a 200µm sieve and transferred to the respective treatment.

2.3 Experimental design and setup

Three separate experiments were carried out to assess the influence of various microalgal diets and their combinations on major parameters related to *A.grani* culture productivity, i.e. (1) egg hatching rate, (2) egg production rate and (3) population increase over a 12 day culture period.

Microalgal diets* used:

Diet 1: *Rhodomonas marina* (Rho)

Diet 2: *Rhodomonas marina* (Rho) + Tahitian strain of *Isochrysis* sp. (T-Iso)**

Diet 3: *Tetraselmis suecica* (Tet)

Diet 4: *Tetraselmis suecica* (Tet) + Tahitian strain of *Isochrysis* sp. (T-Iso)**

*Algae concentration was estimated from Strathmann (1967)

**The combined diets, the proportion is 1:1

All experiments and acclimatization were carried out under similar conditions: seawater 1µm filtered - UV treated; salinity 38±1psu; 24±0.5°C; photoperiod 12L:12D. Algae concentration was estimated from Strathmann (1967).

2.3.1 Acclimatization to different diets

Approximately 200 *A. grani* adults, collected from a stock culture tank, were acclimatized to each diet for 3 days in 5L aquarium with seawater and gentle aeration.

Copepods were fed daily morning and afternoon in excess with the designated algal diets to acclimatize them to respective diets and to remove any potential residual effects of previous diets. Combined use of microalgae was in the proportion of 1:1. Microalgal concentrations were determined daily using a haemocytometer under a microscope (Zeiss – Axioskop 2 - plus).

2.3.2 Egg Hatching Rate experiment (EHR)

As in population increase experiment, the copepods had a 3 days period of acclimatization to the different tested diets. At that time, with the sieve of 200µm adults were collected to a new beaker to ensure that the eggs used in the experiment had a maximum of 24 hours. Freshly produced eggs from each dietary treatment were then carefully collected with a 55µm sieve and total number of eggs was counted with *Sedgwick-Rafter* chamber.

Afterwards, eggs from each treatment were randomly distributed into 100ml beakers. Five replicate beakers *per* diet were used, containing 40 to 60 eggs (counted under a microscope).

Egg hatching success was estimated for each microalgal diet by, calculating the difference between the number of eggs unhatched and the hatched nauplii, at 10, 20, 24, 30 and 48 hours after incubation.

2.3.3 Egg Production experiment (EP)

Following three days of acclimatization, groups of 5 mature *A. grani* females were randomly captured using a broad-tipped pipette and distributed into twelve 100ml beakers, with groups of 3 beakers assigned to each diet treatment as replicates.
After 24 hours *A. grani* females were removed from the beakers and eggs they produced were counted using a *Sedgwick-Rafter* counting cell and a microscope (Zeiss – Axioskop 2 - plus).

Following the procedure described above, new females were randomly selected daily from the stock culture and transferred into a new set of twelve 100ml beakers containing fresh filtered seawater and algal diets to obtain individual 24h egg output for each tested diet of 3 consecutive days.

The daily replacement of females ensures that the new females are fertilized and healthy, being ready to the egg production experiment.

2.3.4 Population Growth experiment

After 3 days of acclimatization, 12 healthy (actively swimming and intact appendages) adults (4 Males and 8 Females) were transferred into 600ml beakers with 300ml seawater and without aeration. A total of 20 replicate beakers were established with 4 replicates for each treatment.

Every morning 30% of the culture medium was exchanged with siphon 55µm mesh to prevent the loose of eggs or nauplii. After water exchanged, a sample was collected to determined microalgal concentrations on the water column, and readjust if necessary the 1500µgC.L^{-1}, a carbon concentration known to saturate copepod feeding (Kiørboe *et al*. 1985). Carbon concentrations were calculated according to Strathmann (1967). When *A. grani* were fed with a binary diet, carbon concentration was divided equally between the 2 algae offered based on their biomass. Feeding was done every morning and afternoon because *A. grani* is a suspension feeder, therefore it is important to ensure that the algae stay in suspension in the water column.

After 12 days, content from each beaker was drained through a 55µm sieve and all eggs, nauplii, copepodites and adults retained were fixed with 4% formaldehyde and stored 4°C. The counting of *A. grani* samples were made using a *Sedgwick-Rafter* cell counter and a microscope (Zeiss – Axioskop 2 - plus).

The specific population growth rate (*K*) of *Acartia grani* was calculated using the following formula (Omori and Ikeda, 1984; Hada and Uye, 1991):

$$K = (ln N_t - ln N_0)/t$$

Here, *t* is the culture days (12) and N_0 and N_t are the initial and final density of copepods, respectively.

In addition, doubling time (D_t) was calculated by dividing $\log_e 2$ by the populations growth rate (*K*) according to the following formula (James and Al-Khars, 1986):

$$D_t = (\log_e 2)/K$$

2.4 Statistical analysis

Data from all experiments were analyzed using one-way ANOVA. When significant differences (*p*<0.05) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments (*p*<0.05). All statistical analyses were conducted using SPSS, version 20.0. Data are presented as mean±standard deviation (SD).

3 Results

3.1 Egg Hatching Rate (EHR)

Figure 1 present the influence of microalgae diet on *Acartia grani* egg hatching experiment (EHR). Twenty four hours after incubation, hatching rate was highest on the binary algal diet Rho+T-Iso, with a mean EHR of 13.5%. That tendency continued until the end of the experiment. Compared to 24h EHR, 48h hatching rates improved across all dietary treatments, though Rho+T-Iso diet continued to present higher EHR (38.5%), while the remaining dietary treatments had very similar EHR (Rho: *24.8*%; Tet: *25.4*%; Tet+T-Iso: 22.9%). At the end of experiment (72hours) the mono-diets, Tet and Rho showed the lowest EHR, 26.9% and 29.6% respectively. The highest rates were found in binary diets Rho+T-Iso (44.2%) and Tet+T-Iso (35.1%).
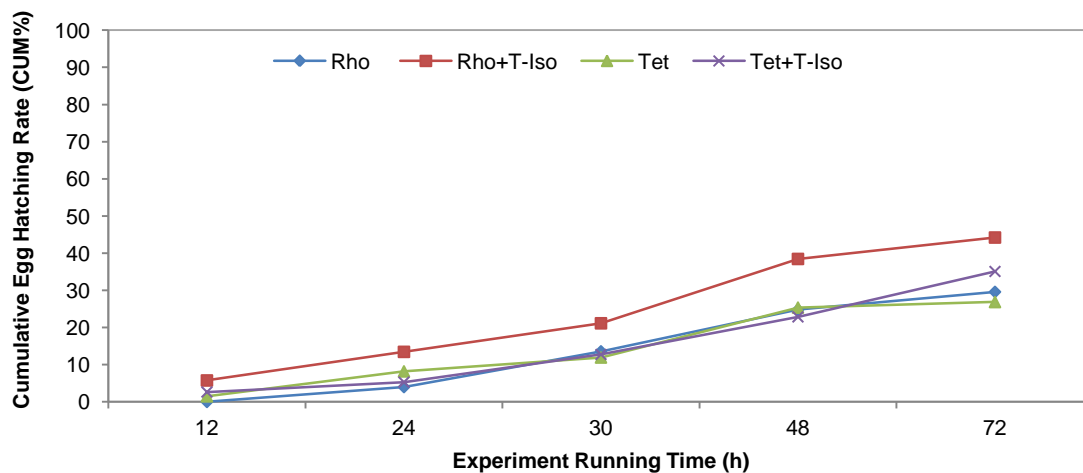
Figure 1 – The cumulative egg hatching rate (CUM%) of *Acartia grani* eggs produced by adults fed four different microalgae diets, over 72 hours. Eggs were incubated under identical condition of 24±0.5°C, 38±1 psu and photoperiod 12L:12D.

3.2 Egg Production (EP)

The egg production (eggs female^{-1} day^{-1}) of the different microalgae diets during 3 consecutive days are presented in Figure 2. Microalgal diets significantly influenced ($p<0.05$) egg production (EP) of *Acartia grani* on day 1. Rhodomonas treatment was significantly higher (4.7± 0.9) than egg ouput of Tetraselmis diet (1.8±0.4).
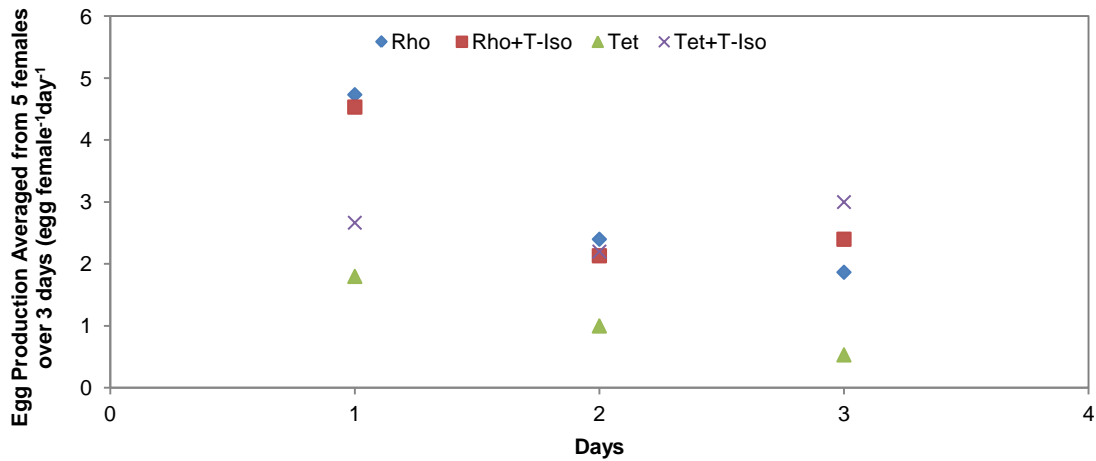
Figure 2 – Mean egg production (eggs female^{-1}day^{-1}) of *Acartia grani*, eggs produced by adults fed four different microalgae diets (Rho, Rho+T-Iso, Tet, Tet+T-Iso), on the 3 experimental days. Data are presented as mean±SD. (Day 1 p=0.01; Day 2 p=0.82; Day 3 p=0.62).

On the remaining days no significant differences were found between treatments. Still, dietary treatment of Tetraselmis presented in all three days the lowest egg production per female per day.

Clearly the presence of *Rhodomonas* algae affected the EP. When calculated the overall mean 24h EP (Table I), the egg production was significantly higher (p<0.05) in the Rho dietary treatment (3.66±1.40) followed by binary diet (Rho+T-Iso) with an EP of 3.30±1.34. The lowest EP was found in *Tetraselmis* treatment, which is in accordance with the Figure 2, being significantly lower than Rho+T-Iso and Rho treatments, except that of Tet+T-Iso (2.62±1.33).

Table I – Effects of diets on averaged 24h egg production of *Acartia grani*. For each treatment, 24h egg production was averaged from 5 females over 3 days. Data are presented as mean±SD. (One way ANOVA p=0.023).

Diet	Eggs female^{-1}day^{-1}
Rho	3.66 ±1.40[a]
Rho +T-Iso	3.30 ±1.34[a]
Tet	1.40 ±0.84[b]
Tet +T-Iso	2.62 ±1.33[ab]

3.3 Population Growth

The results showed that diet significantly affected the population growth of *A. grani* after 12 days of culture on different microalgae diets. The averaged final population numbers of *A.grani* are presented in two categories, i.e. "All Stages included (i.e. including eggs); "All Post-Egg-Stages" (i.e. excluding eggs) (Fig. 3). Highest mean final population was observed for *A. grani* fed on diet Rho+T-Iso (1783.0±560.6) if all stages were considered. In fact, Tukey´s test reveled significance differences between this dietary treatment and *Tetraselmis*, which presented the lowest mean population increase (325.0±135.7) When analyzed without eggs (all post-egg-stages), the highest mean final population number was observed on adults fed microalgae diet Tet+T-Iso (511.0±94.3), being the second most productive diet followed closely by the Rho+Iso dietary treatment (508.0±114.1). The *Tetraselmis* diet produced the lowest registered results ($p < 0.05$), with a mean final population of only 41.0±16.4 individuals.
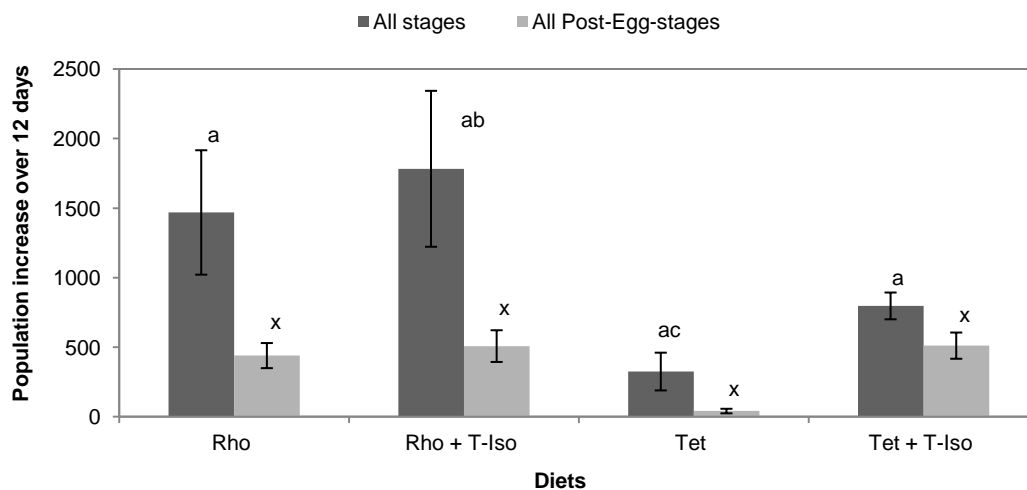
Figure 3- Mean final total population of *Acartia grani* cultured at four different microalgae diets for a 12 day period. Initial population was 8 females: 4 males Data are represented as mean±SD. All Stages bar (all life stages: eggs, nauplii, copepodites, adults); All Post-Egg-Stages bar (nauplii, copepodites, adults). Different letters on the tops of bars indicate significant differences ($p < 0.05$). One way ANOVA: All stages included $p = 0.023$; All Post-Eggs-Stages $p = 0.001$; (Diet 1: *Rhodomonas marina;* Diet 2: *Rhodomonas marina + Isochrysis sp.* (T-Iso); Diet 3: *Tetraselmis suecica*; Diet 4: *Tetraselmis suecica + Isochrysis sp.* (T-Iso).

Analysis of population different stages revealed major differences in the number of eggs between dietary treatments, again with clear positive effects of *Rhodomonas* in egg production. *Tetraselmis* diet produced lower numbers ($p < 0.05$) from the remaining treatments for both copepodite and adult stages.

Table II - Mean number of four life stages (eggs, nauplii, copepodites and adults) within the population of *Acartia grani* cultured for 12 days at four different diets (Rho; Rho+T-Iso; Tet; Tet+T-Iso) from an initial number of 12 adults. (K) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences ($p<0.05$). Data are represented as mean ±SD.

Treatment	Eggs	Nauplii	Copepodites	Adults	K	D_t
Rho	1029.0 ±339.4[a]	107.0 ± 42.1[a]	249.8 ±78.8[a]	83.0 ±47.3[a]	0.39 ±0.02[a]	1.73 ±0.07[a]
Rho +T-Iso	1275.0 ±385.6[a]	285.8 ±136.7[ab]	164.5 ±23.7[a]	57.8 ±19.0[ab]	0.41 ±0.01[a]	1.66 ±0.05[a]
Tet	283.8 ±57.6[b]	32.5 ±37.,1[ac]	6.0 ±5.7[b]	2.5 ±1.,7[b]	0.27 ±0.01b	2.52 ±0.08b
Tet +T-Iso	286.0 ±181.0[b]	260.0 ±138.2[ab]	179.3 ±74.0[a]	72.0 ±36.2[a]	0.35 ±0.02c	1.99 ±0.11c
	p= 0.000	*p*= 0.011	*p*= 0.000	*p*= 0.015	*p*= 0.00	*p*= 0.00

The specific population growth rate (K) of *A. grani* was calculated for all treatments (Table II). It ranged from 0.27 on Tetraselmis diet to 0.41 in Rho+T-Iso. Population doubling time (D_t) demonstrates that Tetraselmis was the dietary treatment that needed more time to grow (2.52±0.08) and that the binary diet (Rho+T-Iso) had the lowest doubling time (1.66±0.05), which is in agreement with previous results. For both K and Dt significance differences were observed between these dietary treatments (p<0.05).

Mortality of the initial adult population was registered and results revealed that on the first three experimental days, between 10% (Tet+T-Iso) and 15% (Tet) of the initial adults had died. By the 10th experimental day, mortality reached 68.8% at *Tetraselmis*, followed by *Rhodomonas* diet with a mean mortality rate of 37.5%. From that day onwards mortality was not counted, due to possible misleading of initial incubated adults with born grown adults during the experiment.

4. Discussion

Calanoids copepods have a non-visual, active raptorial mode of feeding, capturing and ingesting a variety of animal prey (Tiselius and Jonsson, 1990). Successful growth and development of cultured species depends largely on the nature and content of biochemical constituents in the food provided. These constituents are used in the anabolic process for tissue production, in catalyzing metabolic process and in the creation of energy to power those processes (Matias-Peralta et al., 2012).

This study evaluated the best diet to optimize the culture of *Acartia grani*. In all current experiments, *A. grani* were acclimatized to experimental diets for 3 days, period judged to be sufficient. Based on our results, the use of *Rhodomonas* algae affected positively all of the analyzed parameters, performing better than *Tetraselmis*. Moreover, our results show that the use of the binary diets seems to more appropriate, positively

affecting population dynamics of *Acartia grani*. Rho+T-Iso dietary treatment clearly presented a superior egg hatching rate over the 72 hours experiment, followed by diet Tet+T-Iso. Several authors (Koski et al., 1998; Broglio et al., 2003, Tang and Taal, 2005), refered the high nutritional value of these microalgae for copepods. Knuckey et al. (2005) demonstrated the superiority of *Rhodomonas sp.* as a mono-algal diet for *Acartia sinjiensis*. However, bear in mind that the use of *Rhodomonas* in a commercial aquaculture operation is limited by the instability of this species in mass culture. Cultures can fail to initiate exponential growth or prematurely enter stationary phase. Cultures also have a short stationary phase compared to other microalgae commonly used in aquaculture and can quickly die (Knuckey et al., 2005).

On the other hand, *Tetraselmis* monoalgal diet did not seem suitable for *Acartia grani*. When compared to other diets, results were lower in most experiments, which is in accordance with several other authors that reported the same issue, i.e., Camus et al. (2009) for *Bestiolina similis*; Milione and Zeng (2007) in *Acartia sinjiensis*; and Koski et al. (1998) for *Pseudocalanus elongatus*.

Egg production is one of the principal factors determining copepod culture productivity and has been linked to the maternal nutrition (Castro-Longoria, 2003). Averaged 24h egg production was favorable again for the treatment that had *Rhodomonas* included. In monoalgal treatment (Rho) the averaged egg production of 4 eggs female^{-1}day^{-1} for *Acartia grani* was similar to values observed by Koski et al. (1998) for *Pseudocalanus elongates* (5 eggs female^{-1}day^{-1}), although Broglio et al. (2003) found for the same concentration of *Rhodomonas* (1500µgCL^{-1}) a value of 25 eggs female^{-1}day^{-1} for *Acartia tonsa* and McKinnon et al. (2003) found that *Rhodomonas* supported high egg production rates of *A. sinjiensis* (up to 33 eggs female^{-1}day^{-1}).

Analysis of population growth is probably more pertinent to the ultimate goal of improving productivity of copepod culture for hatcheries because it provides a summary of the dietary effects on a range of inter-related parameters, including egg production, egg hatching rates, nauplii and copepodite development time and survival (Milione and Zeng, 2007). Diet significantly affected the population growth of *A. grani* in our study. The binary diet Rho+T-Iso presented great growth when all stages were included with 1783 individuals, followed by the monoalgal treatment Rho (1469 individuals), exclusion made when analysis was performed on all post-egg stages where the binary diet Tet+T-Iso presented the highest population increase. This high growth can be related to the *Isochrysis sp.* microalgae, which is considered among the best food sources for filter-feeders, because of their high content of essential fatty acids that promote high survival and growth (Watanabe et al., 1983; Brown et al., 1989). On other hand, *Tetraselmis* microalgae has been reported to induce a high degree of deformities and complete lack of

development beyond the copepodite stage in *A. sinjiensis* (Knuckey et al., 2005). Another point of view for poor performance of *Tetraselmis* was the bad digestibility of this microalgae (Puello-Cruz et al., 2009). Moreover during the experiments, we observed that *Tetraselmis* deteriorates the quality of water relatively fast, and it is possible to see a superficial layer in water surface, that sometimes adheres to swimming appendages of copepods leading to death.

When different stages were analyzed separately and in concurrence to our previous results of egg production and egg hatching rate, the population growth in the Rho and Rho+T-Iso had the highest number of eggs over the 12 days of experiment, (1029 and 1275, respectively). We also found that diets with T-Iso included had a high number of nauplii in concordance to what Knuckey et al., (2005) reported, that T-Iso supported better nauplii development.

The poor performance of *Tetraselmis* was evident in adult's stages over the 12 days, since final population was only of 3 individuals. This microalgae was responsible for high mortality, after 3 days of culture 15% of the adults died, and by the 10th experimental day, mortality reached 68.8%, which was crucial for the bad development of population.

5. Conclusion

Of the many algal species that have been used in aquaculture, *Rhodomonas* and *Isochrysis* have been conspicuously successful as a food for rearing copepod species (Støttrup et al., 1986; Lacoste et al., 2001; Rippingale and Payne, 2001; Lee et al., 2006).

In summary the presence of *Rhodomonas* microalgae clearly affect the development of *Acartia grani*, although the *Isochrysis* seems to be important complement algae in early stages, because of is nutritional value and maybe the small size of the cells. It is recommended that for the culture of *Acartia grani* the binary diet of *Rhodomonas* + *Isochrysis* is used to achieve a good culture production.

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Egg production, egg hatching success and population increase of the calanoid copepod, *Acartia grani* (Calanoida: Acartiidae), using various combinations of autotrophic and heterotrophic protists

1. Introduction

Marine pelagic systems are characterized by the presence of a wide variety of autotrophic and heterotrophic species, which represent potential food items for omnivorous copepods. In the last two decades, appreciation for the importance of large heterotrophic protists (ciliates and dinoflagellates) in the copepod diet has grown, because they have been proposed as an intermediate link between the microbial loop and higher trophic levels (Verity and Paffenhöfer 1996; Klein Breteler et al., 1999).

Several studies showed that copepods can ingest protozoa (Gifford and Dagg, 1991; Atkinson, 1994; Levinsen et al., 2000) at higher rates than phytoplankton (Dolan, 1991; Fessenden and Cowles, 1994; Zeldis et al., 2002), and may also preferentially select the former (Stoecker and Sanders, 1985; Stoecker and Egloff, 1987; Sanders and Wickham, 1993; Verity and Paffenhofer, 1996). Thus, the nutritional content of heterotrophic protists is equally, if not more, important as that of algae in regulating zooplankton growth and production (Tang and Taal, 2005), as some heterotrophic protozoa appear to provide essential copepod growth compounds that are not always found in phytoplankton (Klein Breteler et al., 1999). Tang et al. (2001) showed that heterotrophic protists as a trophic link between poor nutritional quality algae and copepods resulted in higher egg production and egg hatching success. Nowadays there is evidence that protozoa hold a key position and are an essential link in pelagic food webs (Calbet, 2008). As predators, heterotrophic protists consume, assimilate and repackage not only the biomass and nutrients of their prey, but, most importantly, upgrade their biochemical constituents, such as the long-chain n-3 essential fatty acids (LCn-3 EFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and sterols (Ackman et al., 1980; Sargent et al., 1987, 2002).

Oxyrrhis marina is an extensively studied heterotrophic flagellate (Montagnes et al., 2011), that exhibits a wide geographic distribution (Watts et al., 2011), easily recognized (e.g. Dodge, 1982) and easy to isolate from the natural environment (Lowe et al., 2011). Klein Breteler et al. (1999) reported that this heterotrophic dinoflagellate grown on the EFA deficient alga *D. tertiolecta* supported rapid growth of the copepods *Temora longicornis* and *Pseudocalanus elongatus* from naupliar stages to adulthood. Calanoid

copepods (e.g. *Acartia grani*) are considered opportunistic omnivores (Turner, 1984; Kleppel, 1993) and have been proven to ingest the dinoflagellate *Alexandrium minutum* (Calbet, 2003). Also, the calanoid copepod *Pseudocalanus* has been successfully reared on the heterotrophic dinoflagellate *Oxyrrhis* (Klein Breteler et al., 1999).

We have also learned that copepods can grow better on mixed-food diets than on single-food diets (e.g., Stoecker and Egloff, 1987; Kleppel and Burkart, 1995; Bonnet and Carlotti, 2001, Camus et al., 2009). Although comparative studies of the suite of effects of autotrophic, heterotrophic and mixed diets on the ingestion, growth and egg survival exist (e.g., Adrian and Frost, 1993; Kleppel and Burkart, 1995; Sanders et al., 1996; Koski et al., 1998; Bonnet and Carlotti, 2001), they remain relatively few. Yet, this comparative approach is useful in linking the functional and numerical responses, and hence, in determining the suitability of different diets for copepods. This approach is also helpful in understanding variability in coupling between primary and secondary production

Based on previous results (Chapter IV) we decided to evaluate the effects of using *Rhodomonas marina* and Tahitian strain of *Isochrysis sp.* (the two algae that stimulated best growth performance of *Acartia grani*) combined with *Oxyrrhis marina*.

The purpose of this experiment was to identify an optimal diet for culturing *A.grani*, with microalgae and dinoflagellates, to achieve maximum productivity of this copepod.

2. Material and methods

2.1 Microalgae culture

All of the microalgae utilized in present experiments are common used algal species in aquaculture, therefore relatively easy to culture with the exception of *Rhodomonas marina*. Two algal species were used in this study: *Rhodomonas marina* (Rho); Tahitian strain of *Isochrysis sp.* (T-Iso). *Rhodomonas* was cultured according to the conditions and maintenance protocols described in chapter 1.3.2. *Isochrysis sp.* (T-Iso) was inoculated by starter cultures supplied by IPIMAR (Olhão) and was by batch method with Nutribloom medium (see Annex, Fig.1), at 20±1ºC salinity 25psu, 24h light in 1L carboys, with continuous aeration. Seawater was 1μm filtered and UV irradiated. The algal cultures were in their exponential growth phase when were used for feeding copepods.

2.2 Dinoflagellate culture - *Oxyrrhis marina*

O. marina inocule came from IPIMAR (Olhão, Algarve) and was kept in our laboratory in 300ml erlenmeyer's with; 20±1°C; photoperiod 24 light.

Several experiments were carried out at CMC in order to determine whether O. *marina* could be cultured in our facilities and used as an intermediate trophic link for copepod populations. Prior to establishing a culture protocol it was necessary to test whether cultures of *O. marina* were (1) fed either single or combination diets of the autotrophic cultured algae, (2) best growing temperatures and (3) light cycle.

Based on literature and in the acquired experience we concluded that the best and simple culture technique for *O. marina* in our facilities, was to keep temperature range of 18–20°C under a 24h fluorescent light, with 1µm filtered - UV treated seawater; salinity 25±1psu. The microalgae choice was *Rhodomonas marina*, and culture was supplied once a week with 10-20ml of microalgae culture in the exponential growth phase. Every month all cultures (300ml) were replicated to new erlenmeyer´s, to initiate new cultures.

To increase the production of *Oxyrrhis marina* to be available for this experiment, the same methodology was followed, but feeding of *O. marina* was done every day with *Rhodomonas marina* and replicated to 500ml Erlenmeyer's once a week. Densities of *O.marina* ranged from 4 to 6×10^4 cel.ml^{-1}.

2.3 Copepod stock culture

Stock culture was kept according to the conditions described in chapter 1.2.7. Adults used in this experiment were collected from a parental culture tank with ±12 days, since eggs were incubated. They were collected with a 200µm sieve and transferred to the respectively treatment.

2.4 Experimental design and setup

Three separate experiments were carried out to determine whether *O. marina* could be used single or in combination with two of the autotrophic cultured algae as feed for *A. grani*. Parameters evaluated of *A.grani* culture productivity were: (1) egg hatching rate, (2) egg production rate and (3) population increase over a 12 day culture period.

Diets used in the experiments:
Diet 1: *Rhodomonas marina* (Rho) + *Oxyrrhis marina* (Oxy)

Diet 2: *Oxyrrhis marina* (Oxy)*

Diet 3: Tahitian strain of *Isochrysis sp.* (T-Iso)**

Diet 4: Tahitian strain of *Isochrysis* sp. (T-Iso) + *Oxyrrhis marina* (Oxy)

**Algae concentration was estimated from Strathmann (1967)

*Dinoflagellate concentration was 110µgCL^{-1}.

All experiments and acclimatization were carried out under similar conditions: seawater 1µm filtered - UV treated; salinity 38±1psu; 24±0.5 °C; photoperiod 12L:12D.

2.4.1 Acclimatization to different diets

Approximately 200 *A. grani* adults, collected from a stock culture tank, were acclimatized to each diet for 3 days in 5L aquarium with seawater and gentle aeration. Copepods were fed daily (morning and afternoon in excess with the designated algal diets to acclimatize them to respective diets and to remove any potential residual effects of previous diets. Microalgal and *O. marina* concentrations were determined daily using a haemocytometer under a microscope (Zeiss – Axioskop 2 - plus).

2.4.2 Egg Hatching Rate experiment (EHR)

As in population increase experiment, the copepods had a 3 days period of acclimatization to the different tested diets. At that time, with the sieve of 200µm adults were collected to a new beaker to ensure that the eggs used in the experiment had a maximum of 24 hours. Freshly produced eggs from each dietary treatment were then carefully collected with a 55µm sieve and total number of eggs was counted with *Sedgwick-Rafter* chamber.

Afterwards, eggs from each treatment were randomly distributed into 100ml beakers. Five replicate beakers *per* diet were used, containing 40 to 60 eggs (counted under a microscope).

Egg hatching success was estimated for each diet by, calculating the difference between the number of eggs unhatched and the hatched nauplii, at 10, 20, 24, 30 and 48 hours after incubation.

2.4.3 Egg Production experiment (EP)

Following three days of acclimatization, groups of 5 mature *A. grani* females were randomly captured using a broad-tipped pipette and distributed into twelve 100ml beakers, with groups of 3 beakers assigned to each diet treatment as replicates.

After 24 hours A.grani females were removed from the beakers and eggs they produced were counted using a *Sedgwick-Rafter* counting cell and a microscope (Zeiss – Axioskop 2 - plus).

Following the procedure described above, new females were randomly selected daily from the stock culture and transferred into a new set of twelve 100ml beakers containing fresh filtered seawater and algal diets to obtain individual 24h egg output for each tested diet of 3 consecutive days.

The daily replacement of females ensures that the new females are fertilized and healthy, being ready to the egg production experiment.

2.4.4 Population Growth experiment

After 3 days of acclimatization, 12 healthy (actively swimming and intact appendages) adults (4 Males and 8 Females) were transferred into 600ml beakers with 300ml seawater and without aeration. A total of 20 replicate beakers were established with 4 replicates for each treatment.

Every morning 30% of the culture medium was exchanged with siphon 55µm mesh to prevent the loose of eggs or nauplii. After water exchanged, a sample was collected to determine *O. marina* and microalgal concentrations on the water column, and readjust if necessary the $110\mu gCL^{-1}$ ($500cel.ml^{-1}$) and $1500\mu gCL^{-1}$ respectively. Carbon concentrations were calculated according to Strathmann (1967).

Feeding was done every morning and afternoon because *A.grani* is a suspension feeder, therefore it is important to ensure that the algae stay in suspension in the water column and that no difference is found between the algae availability and the dinoflagellate.

After 12 days, content from each beaker was drained through a 55 µm sieve and all eggs, nauplii, copepodites and adults retained were fixed with 4% formaldehyde and stored 4°C. The counting of *A.grani* samples were made using a *Sedgwick-Rafter* cell counter and a microscope (Zeiss – Axioskop 2 - plus).

The specific population growth rate (*K*) of *Acartia grani* was calculated using the following formula (Omori and Ikeda, 1984; Hada and Uye, 1991):

$$K = (ln\mathrm{N}_t - ln\mathrm{N}_0)/t$$

Here, t is the culture days (12) and N_0 and N_t are the initial and final density of copepods, respectively.

In addition, doubling time (D_t) was calculated by dividing $\log_e 2$ by the populations growth rate (K) according to the following formula (James and Al-Khars, 1986):

$$D_t = (\log_e 2)/K$$

2.5 Statistical analysis

Data from all experiments were analyzed using one way ANOVA. When significant differences ($p<0.05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments ($p<0.05$). All statistical analyses were conducted using SPSS, version 20.0. Data are presented as mean±standard deviation (SD).

3. Results

3.1 Egg Hatching Rate (EHR)

The results of the hatching rate experiment are presented in Figure I. Overall the egg hatching rate was very low, not reaching 50% until 48h after incubation in all dietary treatments tested. Twelve hours after incubation, lowest results were found on Oxy treatment (0.4%), followed by Rho+Oxy treatment (0.8%). That same tendency continued until the end of the experiment and at 48 hours the EHR was highest in T-Iso diet (30.4%).
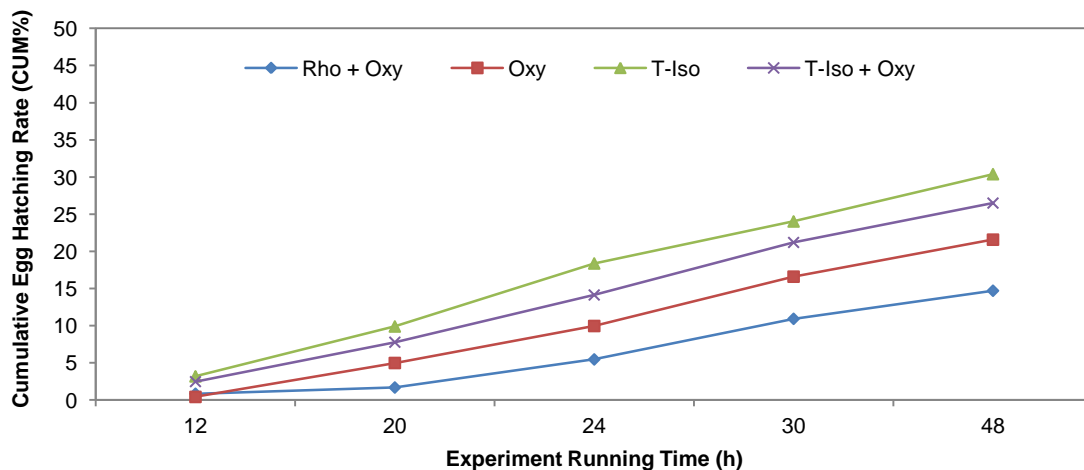
Figure 1 – The cumulative egg hatching rate (CUM%) of *Acartia grani* eggs produced by adults fed four different microalgae diets, over 72 hours. Eggs were incubated under identical condition of 24±0.5°C, 38±1 psu and photoperiod 12L:12D.

3.2 Egg Production (EP)

When fed with autotrophic and heterotrophic protists, significant differences ($p<0.05$) were detected on the EP (eggs female^{-1}day^{-1}) of *A.grani* within each diet treatment in day 1 and day 3 (Fig.2). Egg output was highest (3.7±0.6) with females fed the binary diet Rho+Oxy on day one. By contrast, on the same day, none of the females fed on T-Iso+Oxy diets produced eggs. Similarly, on day three, both females fed on *Oxy* and T-Iso diets did not produce eggs, which evidently contributed for the fact that Rho+Oxy treatment was significantly higher than other diet treatments ($p<0.05$).
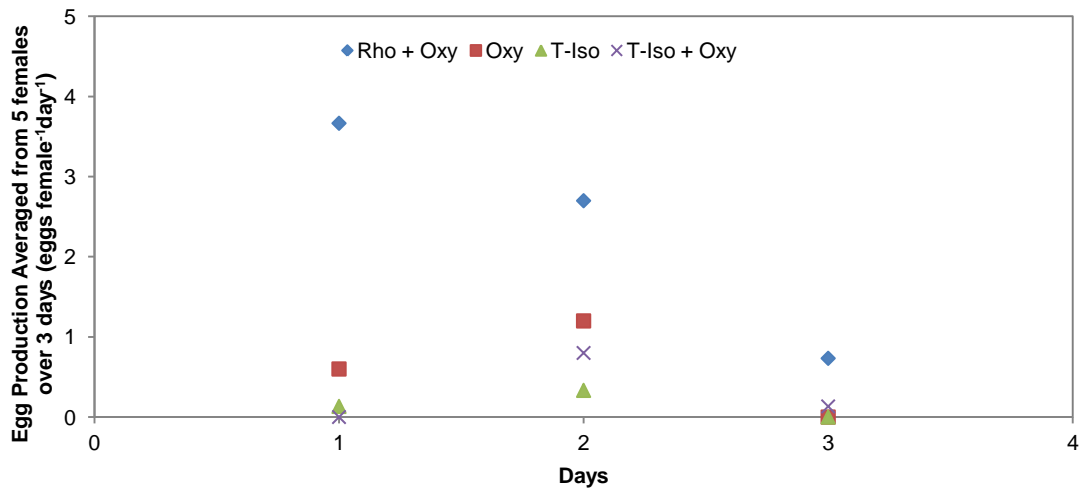
Figure 2 – Mean egg production (eggs female^{-1}day^{-1}) of *Acartia grani*, eggs produced by adults fed four different microalgae diets (Diet 1: *Rhodomonas marina* + *Oxyrrhis marina*; Diet 2: *Oxyrrhis marina*; Diet 3: *Isochrysis sp.* (T-Iso); Diet 4: *Isochrysis sp.* (T-Iso) + *Oxyrrhis marina*), on the 3 experimental days. Data are presented as mean±SD. (Day 1 $p=0.04$; Day 2 $p=0.06$; Day 3 $p=0.00$).

When calculated the overall mean 24h EP (Table I), the egg production was highest in binary diet (Rho+Oxy) with a mean egg production of 2.5±1.7 per female, though no significance differences were found between dietary treatments ($p>0.05$). The lowest EP was produced by T-Iso (0.4±0.10) treatment which was similar to Oxy (0.9±0.6) and T-Iso+Oxy (0.9±0.5) treatments.

Table I – Effects of diets on averaged 24h egg production of *Acartia grani*. For each treatment, 24h egg production was averaged from 5 females over 3 days. Data are presented as mean±SD. (One way ANOVA: p=0.00).

Diets	Eggs female^{-1}day^{-1}
Rho + Oxy	2.5 ±1.7[a]
Oxy	0.9 ±0.6[a]
T-Iso	0.4 ±0.1[a]
T-Iso + Oxy	0.9 ±0.5[a]

3.3 Population Growth

After 12 days of culture on different algal diets, the average final population numbers of *A. grani* are presented in two categories, i.e. 'All Stages Included' (i.e. including eggs) and 'All Post-egg-stages' (i.e. excluding eggs) (Fig.3). Though no significant differences were found between dietary treatments, mean population growth of *Acartia grani* all stages included was highest for the binary diet Rho+Oxy, with a final population of 1795±451.8 individuals, followed by T-Iso+Oxy (1103±353.6). Lowest population was found on T-Iso treatment with only 439±159.9 individuals.

All Post-eggs-stages counts resulted in sustainably reduced final population number as eggs represented the highest counts among all life stages in all dietary treatments.
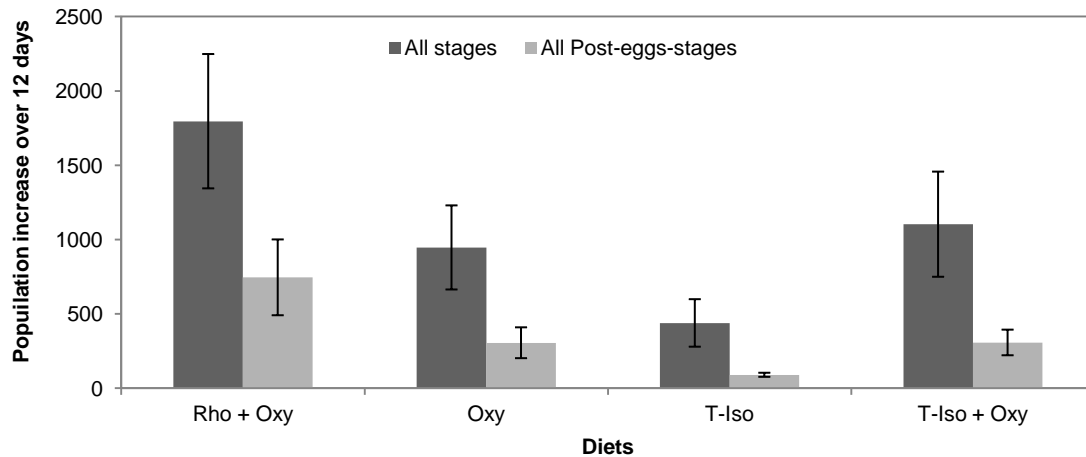
Figure 3- Mean final total population of *Acartia grani* cultured at four different microalgae diets for a 12 day period. Initial population was 8 females: 4 males. Data are represented as mean±SD. All Stages bar (all life stages: eggs, nauplii, copepodites, adults); All post-egg-stages bar (nauplii, copepodites, adults). Different letters on the tops of bars indicate significant differences (p<0.05). One way ANOVA: All stages included p=0.062; All Post-eggs-etages p=0.071. (Diet 1: *Rhodomonas marina + Oxyrrhis marina*; Diet 2: *Oxyrrhis marina*; Diet 3: *Isochrysis sp.* (T-Iso); Diet 4: *Isochrysis sp.* (T-Iso) + *Oxyrrhis marina*).

Overall the binary treatment Rho+Oxy presented higher mean final population when all stages were considered, only due to significant differences (p=0.032) in the amount of eggs produced (Table II), which was three times higher than amount of eggs counted in T-Iso treatment. The remaining stages did not present differences between dietary treatments, though the highest number was always observed within Rho+Oxy treatment.

Table II - Mean number of four life stages (eggs, nauplii, copepodites and adults) within the population of *Acartia grani* cultured for 12 days at four different diets (Rho+Oxy; Oxy; T-Iso; T-Iso+Oxy) from an initial number of 12 adults. (K) is the specific population growth rate, and (D_t) is the doubling time. Different letters indicate significant differences (p<0.05). Data are represented as mean±SD.

Treatment	Eggs	Nauplii	Copepodites	Adults	K	D_t
Rho + Oxy	1050.3 ±514.4[a]	541.3 ±514.8[a]	127.8 ±184.4[a]	76.0 ± 64.8[a]	0.41 ±0.03[a]	1.68 ±0.14[a]
Oxy	641.3 ±209.8[ab]	221.3 ±183.7[a]	35.5 ±32.5[a]	48.3 ±17.2[a]	0.36 ±0.01[b]	1.91 ±0.08[a]
T-Iso	348.8 ±94.3[b]	17.0 ±10.0[a]	44.3 ±32.9[a]	28.3 ±8.2[a]	0.29 ±0.01[c]	2.32 ±0.11[b]
T-Iso + Oxy	795.5 ±132.0[ab]	198.8 ±118.9[a]	75.0 ±110.3[a]	33.3 ±27.9[a]	0.38 ±0.01[ab]	1.84 ±0.07[a]
	p= 0.032	p= 0.116	p= 0.642	p= 0.296	p= 0.00	p= 0.00

The specific population growth rate (K) of *A. grani* was calculated for all treatments (Table II). It ranged from 0.29 to 0.41, being lowest in T-Iso diet and highest on Rho+Oxy diet. Accordingly, D_t values (Table II) showed that T-Iso was the treatment that needed more time to double population (2.32±0.11) and the binary diet Rho+Oxy had the lowest value 1.68±0.14. For both K and Dt significance differences were observed (p<0.05).

Sex ratio of *A. grani* after a 12 day culture period was similar between different microalgae diets (Fig. 4). Highest incidence of females was registered in Rho+Oxy treatment (53.3%) followed by T-Iso+Oxy (51.1%). The highest ratio of males was in T-Iso (54.9%) and *Oxy* (52.8%).
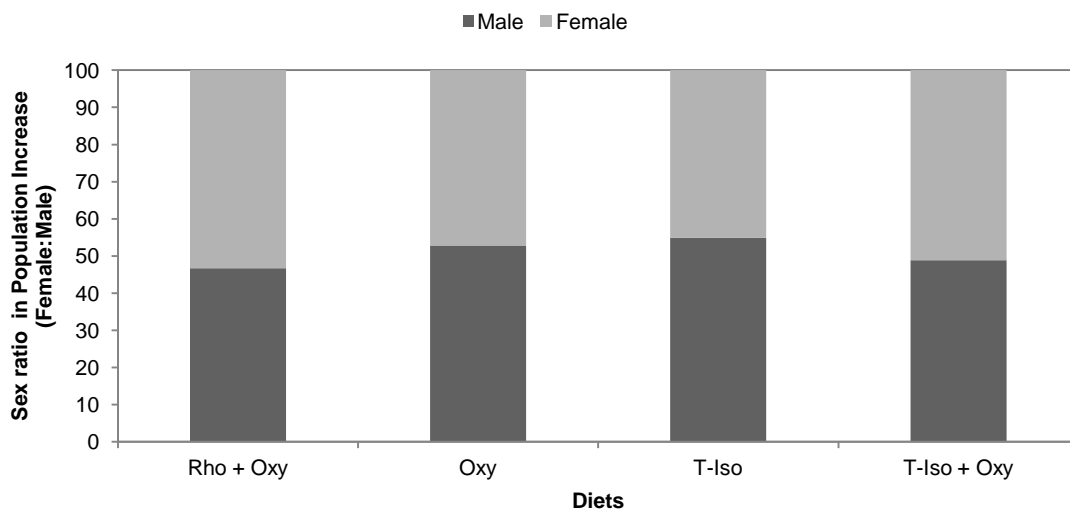
Figure 4 – Sex ratio of *A.grani* in the population increase experiment after 12 days.

Mortality of the initial adult population was registered and results revealed that on the first three experimental days, between 16.7% (T-Iso+Oxy) and 22.9% (Oxy) of the initial adults had died. By the 6th experimental day, mortality reached 41.7% with T-Iso+Oxy treatment, followed by Oxy diet with a mean mortality rate of 39.6%. From that day onwards mortality was not counted, due to possible misleading of initial incubated adults with born grown adults during the experiment.

4. Discussion

Several authors attributed trophic upgrading to modification of algal fatty acid by heterotrophic protists (Klein Breteler et al., 1999; Broglio et al., 2003). The diets of pelagic copepods are characteristically broad (Kleppel, 1993) and strict herbivory of copepods rarely exists in nature. Most copepods prefer feeding on microzooplankton due to their large size, easy perception, as well as the relatively high food quality (Batten et al., 2001; Gifford et al., 2007; Campbell et al., 2009). As copepods are typically omnivorous, any deficiency resulting from a nutritionally poor diet could be compensated in the field by feeding on a wider spectrum of prey (Broglio et al., 2003). Many field studies also showed that heterotrophic protists contribute as much as 100% to a copepod's diet. Thus, the nutritional content of heterotrophic protists is equally, if not more, important as that of algae in regulating zooplankton growth and production (Tang and Taal, 2005).

Based on previous results of experiments performed with different algae diets on *Acartia grani* (Chapter IV) we decided to evaluate the effects of using *Rhodomonas marina* and *Isochrysis sp.* (T-Iso) (the two algae that stimulated best growth performance of *Acartia grani* previously) combined with the heterotrophic dinoflagellate *Oxyrrhis marina*.

In the current study egg hatching rate was highest in treatments with Isochrysis (similar to what we found in our first experiment with Rho+T-Iso and Tet +T-Iso).

Egg production was highest over the 3 days on Rho+Oxy diet, as expected. *Rhodomonas* supports high egg production efficiency and naupliar growth rate (Tang et al., 2001). Contrary to our findings, Tang and Taal (2005) refered for *A. tonsa* that *R. salina* + *O. marina* treatment resulted in trophic downgrading, meaning lower egg production efficiency. In our case the heterotrophic *O.marina* does not downgrade the quality of *Rhodomonas*.

Higher food value is obtained from mixed diets, which are more likely to contain the diversity of biochemical's to satisfy most nutritional requirements for growth (Whyte et al., 1989). In population growth the binary diet *Rho+Oxy* had the highest value of 1795

individuals (all stages included), having the T-Iso treatment the lower population, confirming that *Isochrysis* cannot be provided as monoalgal diet. However, when T-Iso was complemented with *O. marina*, the diet was the second highest, with 1103 individuals over the 12 days. It is generally assumed that by preying on heterotrophic protists a copepod can diversify its diet and obtain a more balanced nutrition (Kleppel, 1993). In the mono diet with *Oxyrrhis marina*, the results were overall good, with 641 eggs, 221 nauplii and 48 adults. The population had a good development with this diet, although total number of individual (946 individuals) was lower than the binary diets.

Analyzing all stages separately between treatments in population growth, the binary diets (Rho+Oxy; T-Iso+Oxy) had the best egg production (population growth) unlike our previous experiment on egg production with values for T-Iso + Oxy diet of only 0.29 eggs female^{-1}day^{-1}. Still, Rho+Oxy treatment, maintained the highest egg production revealing again as an optimal diet to obtain eggs. Copepod reproduction and subsequent development involve multiple life stages and biological processes for which the best suited diet may vary at different life stages or for different biological functions. On this basis, a single species of microalgae may become nutritionally limiting whereas appropriate combinations of algae are likely to offer better balance of required nutrients (Camus et al., 2009).

Overall the binary diet Rho+Oxy was the most complete in all stages (i.e. eggs, nauplii, copepodites, adults) despite of the egg hatching rate that was the lowest of all treatments. This diet proves to be the most complete over the four diet treatments, being a good choice for the production of *Acartia grani*.

5. Conclusion

In conclusion, the present study showed that all the microalgal food were able to support the production of *A.grani*, but clearly the copepod have preferences and a better production with a Rho+Oxy diet was observed. To achieve improved productivity of *A.grani* we suggest that using the binary diet Rho+Oxy was the best option. However and for further studies, a tri-algal diet (Rho + Oxy + T-Iso) probably will improve the production of *Acartia grani*, being more complete for all stages, due to their different requirements.

6. References

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Lipid and fatty acid composition of *Acartia grani* adults fed *Rhodomonas marina* and *Oxyrrhis marina*

1. Introduction

Besides being the natural live prey for marine larval fish, copepods are a rich source of phospholipids, essential highly unsaturated fatty acids (HUFA), natural antioxidants and other essential compounds (Kraul et al., 1992; Sargent et al., 1997). It is generally accepted that copepods can meet the nutritional requirements of fish larvae and that the nutritional superiority of copepods for marine fish larvae to traditional live food such as rotifers *Brachionus plicatilis* and *Artemia* nauplii is well-established (Sargent et al., 1997; Støttrup and Norsker, 1997; Næss and Lie, 1998). Marine larvae of carnivore fish species are generally believed to exhibit high requirements for n-3 HUFA (Yone and Fuji, 1975; Watanabe, 1982; Koven et al., 1990; Rainuzzo et al., 1992; Sargent et al., 1999), especially docosahexaenoic acid (22:6n-3 - DHA) and eicosapentaenoic acid (20:5 n-3 - EPA) which are found to be of particular importance (Watanabe, 1988, 1993; Izquierdo et al., 1989; Estevez and Kanazawa, 1996). Moreover, these essential lipids, EPA, DHA and sterols, have a wide range of critical functions including being important structural components and precursors to bioactive molecules such as eicosanoids and steroids (Ackman et al., 1980; *Sargent* et al., 1987, 2002).

Content of n-3 HUFA in traditional live food is low, unless they are fed diets rich in n-3 HUFA (Watanabe et al., 1983; Léger et al., 1986, 1987; Léger and Sorgeloos, 1991; Olsen et al., 1993; Dhert et al., 1993; Rainuzzo et al., 1994; Evjemo et al., 1997). On the other hand, several coastal copepods have a high content of both DHA and EPA (60% of total fatty acids) (Sargent and Henderson, 1986; Fraser et al., 1989; Evjemo and Olsen, 1997), and their nutritional feasibility judged by larval growth rate, survival, pigmentation and successful metamorphosis has been documented (Holmefjord et al., 1989; Næss et al., 1995; Næss and Lie, 1998; Shields et al., 1999). Though Watanabe *et al.* (1983) reported that culture media did not influence the copepod chemical composition, several authors have reported that levels and ratios of fatty acids in copepods reflected the culture diet (Sargent and Falk-Petersen, 1988; Græve et al., 1994; Delbare *et al.* 1996) and often reflect the lipid composition of algae which varies between taxonomic groups (Chuecas and Riley, 1969; Sargent and Falk-Petersen, 1988), the stage of development of the algal culture (Fernández-Reiriz et al., 1989) and on the stage of development of the copepods (Sargent and Falk-Petersen, 1988).

Calanoid copepods are unable to elongate and desaturate 18:3*n*-3 to produce significant amounts of longer chain HUFA. Norsker and Støttrup (1994) discovered that the harpacticoid copepod *Tisbe holothuriae* does have the ability to elongate and desaturate the 18:3*n*-3 fatty acid supplied by *Dunaliella tertiolecta* to produce significant amounts of the long-chain EFA's, EPA and DHA. Similarly, Watanabe et al. 1978 had found that another copepod, *Tigriopus sp.*, contained high levels of *n*-3 HUFA (12% DHA and 7% EPA) in its lipids even when fed exclusively with baker's yeast.

As important prey items for larval fish, information on the effects of diet on copepod biochemical composition is necessary to better understand fish recruitment and yield. The aim of the present study was to obtain information on the nutritional value of coastal copepod *Acartia grani* which may be used to rear marine fish larvae, and in particular it's content of n-3 HUFA, more specifically DHA and EPA.

Rhodomonas marina was the microalgae utilized, not only because of its nutritional value, rich in proteins and lipids but also because it has been proven to be consumed by *Acartia grani*. *Oxyrrhis marina* is an important nutritional feed for *A. grani*, as predators, heterotrophic protists consume, assimilate and repackage not only the biomass and nutrients of their prey, but, most importantly, upgrade their biochemical constituents, such as the long-chain n-3 essential fatty acids (LCn-3 EFAs), DHA and EPA and sterols (Chu et al., 2009).

2. Material and methods

2.1 Sample collection

Starter culture tanks (500L) were initiated with ±35x10^4 *A.grani* eggs in order to obtain adults for determination of total lipid and fatty acid composition.

Two distinct treatments were prepared. One tank was fed only with *Rhodomonas marina*, and the other with *Oxyrrhis marina*. The concentrations were the same provided in the previous experiments, 1500µgCL^{-1} of microalgae, and 110µgCL^{-1} of dinoflagellate. After 15 days, adults were collected with a 200µm sieve and washed with distilled water and ammonium formate 2%.

Copepods were then gently transferred to cryopreservation tubes and kept at -80ºC (Fryka – Kaltetechnik – Esslingen) until further analysis. The aim was to collect at least 100mg of dry weight of adult's copepods of each treatment.

Samples were then lyophilized (Labconco – Freeze dry system – freezone 4.5) prior to lipid extraction.

2.2 Lipid extraction

Lipids were extracted with a chloroform-methanol mixture (1:2 v/v), containing 0.01% BHT, according to Bligh and Dyer (1959). Briefly, 100-300mg of freeze-dried sample was homogenized with 3ml of chloroform-methanol (1:2 v/v) solution, followed by the addition of saturated NaCl solution. After chloroform and desionized water the sample was ultrasonically extracted during 30min. After filtration the chloroform layer was separated from the methanol-water layer, and dried with anhydrous sodium sulphate. The lipid solution was transferred to a flask and the solvent was evaporated in a rotator evaporator kept at approximately 40ºC. Total lipid (TL) content was determined gravimetrically.

2.3 Transesterification and fatty acid methyl ester analysis

For the determination of fatty acid profiles, methyl esters (FAMEs) will be prepared according to the Lepage and Roy method (1986) modifed by Cohen et al. (1988). The preparation of fatty acid methyl esters is carried out using 5ml of the acetylchloride/methanol reagent (1:19v/v). The reaction is done at 80ºC for 1 hour. After cooling, 1ml of water and 2ml of n-heptane is added to the mixture, stirred and centrifuged. The organic phase is collected, filtered and dried with anhydrous sodium sulphate. Solvent is removed under nitrogen and the methyl esters solubilized in 0.1ml of n-heptane. Each sample was made in duplicate. The quantitative analysis is performed in a gas chromatograph (Agilent HP 6890) equipped with a flame ionisation detector and a 5973 Agilent mass selective detector. The separation is performed in a polyethylene glycol capillary column Supercolwax with 30m of length, 0.25mm i.d. and 0.25µm film thicknesses from Supelco. The column is subjected to a temperature program starting at 140ºC for 5min, heating 4ºC min^{-1} at 240ºC. The injector (split ratio 100:1) and detector temperatures are kept constant at 250ºC during the analysis.

Each aliquot was injected in duplicate. The presented results are the average of the values obtained for each sample. Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and/or by the mass spectra. FAME was expressed as mg per g of ground tissue wet weight and as weight percent of the total fatty acids of each sample.

2.4 Statistical analysis

Statistics were performed on positively identified FAs that contain non-zero values in all groups. All groups containing greater than one sample are included. Significant differences in lipid data were confirmed by randomization testing on all data. Where appropriate, samples showing a significant difference were subjected to a Tukey´s multiple comparison test with a significance level set at 95% ($p<0.05$).

3. Results

The diets used in this study were chosen based on previous result of experiments performed with different diets on *Acartia grani* (Chapter IV – V). There were no significant differences ($p>0.05$) in the lipids (%) content of *Acartia grani* adults fed the two diets.

Analysis of the major fatty acids groups - saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) - revealed no significant differences ($p>0.05$) between the two diets. Nevertheless, levels of the fatty acids were superior when *Acartia grani* was fed with the mono diet *Rhodomonas marina*. SFA were dominated by palmitic acid (16:0), followed by stearic acid (18:0).

PUFA dominated the fatty acid composition, accounting for 60% of total fatty acid detected in the Rho diet and 41% in the Rho+Oxy diet. Differences between the two dietary treatments, though no statistically different ($mg.g^{-1}$), were on the expense of SFA of the Rho+Oxy diet. PUFA composition was dominated in both diets by docohexaenoic acid (DHA), followed by eicosapentaenoic acid (EPA). Again, though not statistically different, levels of DHA (22:6n3) were superior on the treatment where *A.grani* were fed with *Rhodomonas marina* ($0.629mg.g^{-1}$).

Levels of DHA/EPA were similar for the two diets: $2.327\pm0.094mg.g^{-1}$ for Rho diet, and $2.176\pm0.060mg.g^{-1}$ for the binary diet.

Table I. Lipid content and fatty acid compositions of total lipid from *A. grani* copepods cultured for 12 days fed *Rhodomonas marina and Rhodomonas marina + Oxyrrhis marina.*

Sample	Rho	Rho + Oxy
Dry weight (mg)	79.933± 0.037	70.850± 0.044
Total lipid (%)	9.396± 2.204	8.652± 0.138
Fatty acids [1]		
14:0	0.112 ± 0.053	0.125 ± 0.001
16:0	0.296 ± 0.102	0.395 ± 0.034
18:0	0.237 ± 0.132	0.223 ± 0.003
Others	0.137 ± 0.063	0.136 ± 0.012
Total SFA	0.781 ± 0.350	0.879 ± 0.025
16:1	0.015 ± 0.005	0.011 ± 0.003
18:1	0.057 ± 0.027	0.053 ± 0.005
20:1	0.015 ± 0.001	0.000 ± 0.000
Total MUFA	0.087 ± 0.033	0.063 ± 0.008
18:2ω6	0.084 ± 0.057	0.075 ± 0.026
18:3ω3	0.201 ± 0.132	0.112 ± 0.017
18:4ω3	0.156 ± 0.111	0.067 ± 0.000
20:4ω6 - AA	0.015 ± 0.005	0.008 ± 0.005
20:5ω3 - EPA	0.274 ± 0.182	0.103 ± 0.009
22:6ω3 - DHA	0.629 ± 0.397	0.228 ± 0.029
Others	0.018 ± 0.012	0.010 ± 0.000
Total - PUFA	1.378 ± 0.895	0.605 ± 0.000
∑ω-3	1.261 ± 0.821	0.511 ± 0.021
∑ω-6	0.117 ± 0.074	0.094 ± 0.021
(ω-3)/(ω-6)	10.706 ± 0.289	5.420 ± 2.561
ω-3 HUFA	0.904 ± 0.579	0.331 ± 0.038
DHA/EPA	2.327 ± 0.094	2.176 ± 0.060
EPA/AA	17.214 ± 6.615	17.016 ± 10.859
Total (mg.g^{-1})	2.231 ± 1.276	1.547 ± 0.017

[1] Results expressed as mg/g of total fatty acid methyl esters. One way Anova for each fatty acid had $p>0.005$.

4. Discussion

As biomarkers, fatty acids (FAs) are versatile tools that are widely used in trophic transfer studies in aquatic food webs (Iverson, 2009). As essential nutrients, FAs play an important role in the growth and survival of many organisms, including marine fish larvae. Marine copepods, the main diet for most marine fish larvae in nature and a number of beneficial effects have been linked to copepod nutrient composition in relation to early larval nutrition. In particular, emphasis has been put on lipid composition, and the content and ratio of the polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) (Scott and Middelton, 1979; Seikai, 1985; Kanazawa, 1993; Reitan et al., 1994; Reitan et al., 1997; Nanton and Castell, 1998; Venizelos and Benetti, 1999; Bell et al., 2003). In fact, DHA levels in wild copepods can be more than 10 times higher than in enriched *Artemia* (McEvoy et al. 1998).

Microalgae are the primary producers of FAs, while zooplankton forms a central link between them and higher trophic levels. Zooplankton consumes, incorporate and modify dietary FAs, and themselves synthesize other FAs. (Parrish et al., 2000; Dalsgaard et al., 2003). On this study we have seen that microalgae and the heterotrophic protists stimulated good growth performance in *A.grani* (Chaper IV and Chapter VI). Knuckey et al. (2005) also demonstrated the superiority of *Rhodomonas* sp. as a mono-algal diet for *Acartia sinjiensis*. Moreover, Kleppel (1993) says that by using a binary diet with protists, it is generally assumed that by preying on heterotrophic protists a copepod can diversify its diet and obtain a more balanced nutrition.

Based on the previous results lipid and fatty acid composition of *A. grani* adults fed on *Rhodomonas* and *Rhodomonas* + *Oxyrrhis* was evaluated and no significance differences (p>0.05) were found between total lipid content and the fatty acids composition of adults fed with the two different diets. Our results showed that fatty acids composition of *Acartia grani* were similar to the composition observed by Støttrup et al. (1999) for *Acartia tonsa* fed with *Rhodomonas*. In general, for the main fatty acids we found slightly inferior values, with the exception of the saturated fatty acids (SFA) that reached the 35.8g/100g of total fatty acids. Levels and ratios of fatty acids in copepods vary depending on the dietary input (Sargent and Falk-Petersen, 1988; Græve et al., 1994) and often reflect the lipid composition of algae which varies between taxonomic groups (Chuecas and Riley, 1969; Sargent and Falk-Petersen, 1988), the stage of development of the algal culture (Fernández-Reiriz et al., 1989) and on the stage of development of the copepods (Sargent and Falk-Petersen, 1988). Tough no direct comparison is possible between composition of other microalgae and copepods used in

our study, we were able to see that Tremblay et al. (2007) found for *Rhodomonas salina* similar SFA contents to those found in *A.grani* copepods. Nevertheless, PUFA content, including DHA and EPA were presented in much higher concentrations in *A. grani*.

Parrish et al. (2012) cultured the harpaticoid copepod *Tisbe furcata* with the same binary diet (Rho+Oxy) that we used in our work. SFA's were higher (55.1%) compared to the 36% of the *T.furcata*. As for monounsaturated acids (MUFA), these were superior (34.4%) in harpaticoid copepod, compared to the 3.9% for *A.grani*. Regarding DHA, PUFA and DHA/EPA fatty acid content of *Acartia grani* we found that it was higher when compared to *Tisbe furcata* content. Moreover, Klein Breteler et al. (1999) cultured *Oxyrrhis marina* fed on *Rhodomonas* and found very similar contents of myristic acid (14:0), palmitic acid (16:0) and EPA to those found in *A.grani*.

5. Conclusion

For all the above said, it is possible to infer the potential of *Acartia grani*, as a good source of fatty acids for marine fish larvae. Further analysis is needed to determine seasonal variations and different life stages fatty acid profile. Furthermore, it would also fundamental to evaluate or *Rhodomonas* strain and Oxyrrhis in order to confirm the existence of trophic upgrading between these two feed source, as did Klein Breteler et al. (1999), and Veloza et al. (2006) who fed *D. tertiolecta* and *Rhodomonas* to separate *O. marina* cultures, and with those of Tang and Taal (2005) and Parrish et al. (2012) who fed *D. tertiolecta*, *I. galbana* and *Tetraselmis* to separate *O. marina* cultures.

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Effect of *Acartia grani* culture density on cannibalism occurrence

1. Introduction

Several abiotic and biotic factors are known to affect copepod culture to be used as live prey for the aquaculture industry. Amongst them, density of individuals is crucially important to the copepods culture. Despite all efforts in the development of a method for the semi-intensive culture of calanoid copepods, little emphasis is still placed on their mass production to commercial levels. Calanoids are mostly used in larviculture and their maximum density in culture is an important limitation. Calanoid copepods are generally believed to have low tolerance to poor water quality (Payne and Rippingale, 2001) therefore their cultivation at high densities is considered difficult due to density-related stress factors (Jepsen et al., 2007). Past studies investigating effects of copepod stocking density on their culture productivity have mainly assessed egg production and egg hatching success and used them as major indicators (Medina and Barata, 2004; Peck and Holste, 2006; Jepsen et al., 2007). However, other biological parameters, such as cannibalism represent other important criteria. Cannibalism rate is generally reported to increase with increased stocking density (Gallucci and Ólafsson, 2007) due to increased encounter rates of individuals in the rearing tanks. Similarly to the wild, in culture tanks, copepod populations consist of various developmental stages, often coexisting in the same water mass. In this case, it is highly likely that adults and later copepodites often encounter their own offspring. Smaller individuals (younger stages) are then more likely to be victims of the more developed stages (Uye and Liang, 1998, Ohman and Hirche, 2001). For example, Hada and Uye (1991) demonstrated that cannibalism rate increased asymptotically with naupliar and copepodite density (as prey) for *Sinocalanus tenellus* and Lazzaretto and Salvato (1992) reported the existence of cannibalistic behavior in *Tigriopus fulvus* females towards first-stage on-ralted nauplii.

Although cannibalism is known to be common in *Acartia spp*. (Mauchline et al., 1998; McKinnon et al., 2003) no work has been done with *Acartia grani*.

The main goal of this experiment was to evaluate the effect of adult stocking density on cannibalism rate toward newly hatched nauplii.

2. Material and Methods

2.1 Experimental design and setup

Newly hatched nauplii (hatched within 24h) of *Acartia grani* were isolated from unhatched eggs by attracting them to a light source utilizing their positive phototoxic behavior. The nauplii collected were subsequently counted and an identical number of 300 nauplii *per* liter were randomly introduced into each of twenty 50ml replicate vessels (5 replicates *per* treatment). Using a 250µm mesh sieve, the predators consisting of late copepodites (C-5) and adults, were pre-isolated from stock culture and suspended in Petri dishes with fresh algae. After nauplii had been distributed into each replicate vessel, the pre-isolated adults and copepodites were then counted and added to each replicate vessel to form 5 densities of 125, 250, 500, 1000 and 2000ind./L, respectively. Fresh *Rhodomonas* was added to all replicates at 1500µgCL^{-1}.

To avoid possible confounding effects of nauplii hatched out from eggs produced by adult females introduced as predators, cannibalism experiment lasted 8 hours. This experimental duration was selected based on previous hatching results (Chapter I) for *Acartia grani* eggs. At the end of the 8h experiment, content of each replicate was collected on 55µm mesh sieve and fixed with 10% formalin. The remaining nauplii were then counted using a dissecting microscope (Zeiss – Axioskop 2 Plus). The difference between initial and final number of the nauplii in each replicate was assumed due to cannibalism, as it is expected that within an 8 hour period, with this culture conditions, nauplii survival would be high(>95%).

2.2 Statistical analysis

Data from all experiments were analyzed using one way ANOVA. When significant differences ($p<0.05$) were found, Tukey's multiple comparisons test was used to determine specific differences among treatments ($p<0.05$). All statistical analyses were conducted using SPSS, version 20.0. Data are presented as mean±standard deviation (SD).

3. Results

Predation of *A. grani* adults and late copepodites on nauplii increased with increasing stocking density (*p*<0.05) (Fig.1). Statistical analysis showed that cannibalism rate on nauplii was significantly lower at the lowest stocking density (125 ind./L) when compared to the remaining treatments. Moreover, significant differences were also found between 250 ind./L density and the highest stocking density of 2000 ind./L (*p*<0.05).
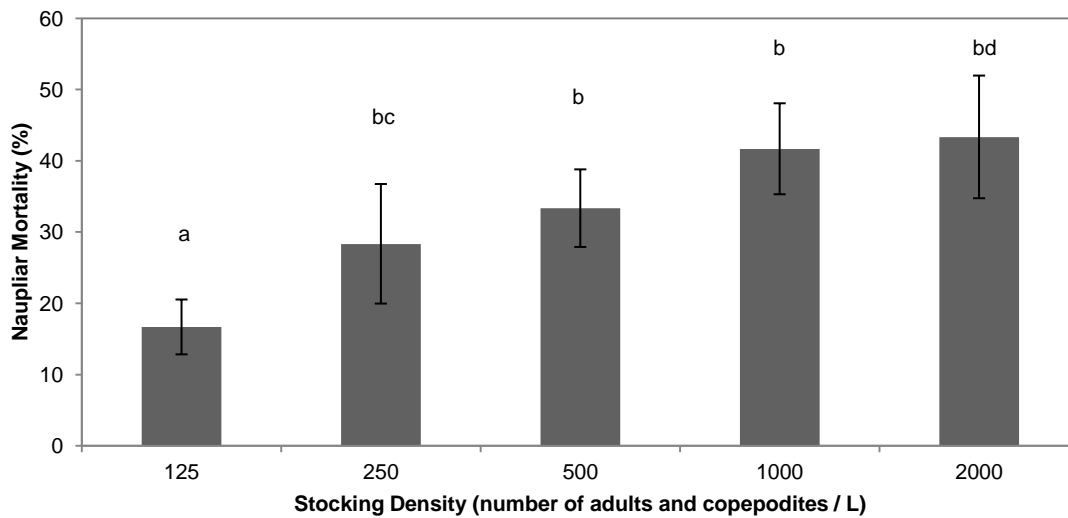
Figure 1 – Naupliar mortality rates at different *Acartia grani* stocking densities. Data are presented as mean±SD. Different letters on the tops of bars indicate significant differences (*p*<0.05).

4. Discussion

Cultures techniques of copepods are to be improved to render the production of copepod reliable and useful to aquaculture businesses. We evaluated the effect of cannibalism of the copepod *Acartia grani* on nauplii, which could significantly impact culture productivity. Cannibalism rate is generally reported to increase with increased stocking density (Gallucci and Ólafsson, 2007). Results from current experiment confirmed a trend of A. *grani* for cannibalism regardless of the existence of other food items as *Rhodomonas*. Significantly higher predation was observed within densities as low as 250 ind./L. Towards the densest predator treatment of 2000 ind./L significant differences were again found when compared to that of the lower density treatments. Findings of our experiment are in accordance to what Camus and Zeng (2009) found for *Acartia sinjiensis* at the same densities.

5. Conclusion

Further investigation should be done in order to investigate the influence of density on population growth to obtain a more complete picture on the various factors contributing to cannibalistic rates of *Acartia grani*. Moreover, it would be interesting to determine cannibalistic rates over eggs, since species belonging to the same genera (*Acartia*) are known to prey upon eggs, eating twice as more eggs than they can produce themselves (Drillet, 2012).

Finally it would also be useful to evaluate the degree to which other food items influence cannibalism. Tough in our case the amount of microalgae supplied to each treatment was enough to meet copepodite and adult demands, due to our methodology it is hard to ascertain whether there was an active selection of prey particle or direct effect of random grazing.

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4| Discussion and Final Remarks

4.1 Final Discussion

Copepods, the most numerous multicellular organisms on earth, are the major food source of numerous species of fish larvae. In 1978, Watanabe et al. analyzed fatty acid content of two marine copepods, *Trigriopus* and *Acartia*, and concluded they presented a high nutritional value for rearing juvenile fish. Years passed and several studies have been performed on copepods, so the amount of knowledge on these organisms has grown. Yet, a lot is still to be "discovered" in this small world of zooplankton.

In aquaculture, they are known to improve fish larval quality and recently proved to be a key for the success of new species, such as Tuna and Grouper. However, due to difficulties in rearing sufficient quantities of copepods and the high associated costs, copepods are today not widely used in the aquaculture industry.

On this work, we focused on the copepod *Acartia grani* because of the known value of the family *Acartiidae* as live feed, and the wide distribution of this specie. The results presented in this work increase the available knowledge about the copepod *Acartia grani*, being accomplished the aim that we proposed to.

Results showed that *Acartia grani* could be produced in all of the experimented biotic and abiotic variables. Still, *A. grani* has its own set of requirements to gauge success and therefore it performed better within optimal sets of parameters.

Observation of parental culture tanks and results found between different experiments also showed that differences between cohorts are evident, thus affecting development of the population. Peck and Holste (2006) referred that using eggs produced from different cohorts (or even from different days from the same cohort) could contribute to variability.

Egg hatching is one of the factors that are related with copepods productivity. In Chapter I we tried to understand the optimal temperature to initiate culture. Highest temperatures seemed to encourage embryogenesis of the egg, resulting in satisfying egg hatching success. If lower temperatures were used then more time was needed until the first nauplii appeared, since the eggs seem to be in quiescence stage (Hansen et al., 2010). Yet, Guerrero and Rodriguez (1998) observed in their study that 18°C temperature seems to be favorable for egg leaving the dormancy phase in *Acartia grani*. As a result of this experiment, 24ºC was the chosen temperature for culturing *A. grani* in all studies performed during this work (Chapter III to Chapter VII).

Comparison between the results of the best egg hatching rates at 48h in all the different tested parameters revealed that EHR was relatively low, but with similar values across experiments. Best EHR (38.5%) at 48hours was observed at 24ºC on the dietary experiment (Chapter IV) using binary diet Rho+T-Iso. Nevertheless, in Chapter I

experiment as soon as 29h after eggs incubation, at the 28°C treatment an EHR of 50% was reached. For this reason, EHR from Chapters II to V were expected to be higher, because of the warmer temperatures used. The eggs from the cohort used in Chapter I seem to response better than eggs used in all the other experiments, including Chapter II, where same temperatures were analyzed and the EHR of 28°C at 30hours was of 4.7%. Rivero (2008) observed that growth rates of *A.grani* are similar to those reported for *A.tonsa* at the same temperature, being the highest growth rate at 26.6°C and 28°C for *A.grani*. Chinnery and Williams (2004) found for species of *Acartiidae* family that nauplii improved survival and developed faster as temperature increased from 5 to 20°C. Takahashi and Ohno (1996) found for *A.tsuensis* the optimum growth was achieved at warm temperatures, around 25°C. Finally, Milione and Zeng (2008) had the same conclusions for *A.sinjiensis* in population growth was significantly higher at warmer temperatures (25-30°C) as compared to lower temperatures (10-20°C). These results show that *Acartia* species seems to have better development in warmer temperatures. However, in my personal observations the treatments with the temperatures of 28°C are very instable because in a short period it promotes the growth of bacteria's, degradation of food and posteriorly the water quality, which in most cases can be fatal for the copepods. For these reason, and despite results of Chapter II experiments, we recommend for optimal production of *Acartia grani* temperatures ranging between 24°C and 26°C.

Apart from egg hatching, egg production is one of the main factors determining copepod culture productivity (Castro-Longoria, 2003), being responsible for the renovation of new copepods. Overall, results of egg production when compared with other studies seemed to be lower than the expected. For the family *Acartiidae*, Broglio et al. (2003) found using the same concentration of *Rhodomonas* ($1500 \mu gCL^{-1}$) a 25 eggs female^{-1}day^{-1} for *Acartia tonsa*, and McKinnon et al. (2003) found that *Rhodomonas* supported high egg production rates of *A. sinjiensis* (up to 33 eggs female^{-1}day^{-1}).

Our best egg production over the days in the different experiments ranged between 3.7±0.6 eggs female^{-1}day^{-1} in the diet Rho+Oxy (Chapter V) to 10.5±1.5 eggs female^{-1}day^{-1} in the 18°C temperature (Chapter II). According to Rodriguez et al. (1995), *Acartia grani* produces eggs continuously, a few at time and not in distinct clutches. Also, several authors have reported that both *Acartia tonsa* (Stearns et al., 1989; Peck and Holste, 2006) and *Acartia grani* (Calbet and Alcaraz; 1996) feeding on high food during the night, produce more eggs than copepods fed on high food during light periods. Though *Acartia* species are known to spawn at night (Mauchline, 1998) and it is known that under constant illumination copepods are probably active 24h around, requiring higher metabolic

rates (Camus and Zeng, 2008), photoperiod experiment (Chapter III) revealed no significant differences in egg production.

However, low egg productions were not reflected in the population increase over the 12 days. With only an initial number of 8 females and 4 males, after 12 culturing days the treatment Rho+T-Iso produced a total of 1275 eggs (Chapter IV) with a final mean population of 1783.0±560.6 individuals. Milione and Zeng (2007) reached a mean population growth of 1091±80 of *A.sinjiensis* individuals after being fed with binary diet Tet+T-Iso over 8 days. For the Calanoida copepod *Bestiolina similis,* Camus et al. (2009) over 12 culturing days achieved in binary diet Tet+T-Iso 541.0±53.6 individuals.

Regarding, mean final number of copepodites and adult stages, our results revealed that best treatments were found when using *Rhodomonas* as the main algae source. In fact, specific population growth rate (*K*) of *A. grani* was better in diet experiments (Chapters IV – V) with Rho+T-Iso and Rho+Oxy treatments. Accordingly, D_t value of *Tetraselmis* diet (Chapter IV) was the treatment that needed more time to double the population D_t=2.52±0.08. In fact, water quality in diets with T-Iso or Tet was very low, actually being in some cases mortal for copepods. However, T-Iso diet presented good results as complement microalgae. Though, Watanabe et al. (1983) considered *Isochrysis* microalgae the best food source for filter-feeders, clearly the cryptophyta microalgae *Rhodomonas marina* is of extremely importance in the culture production. Among microalgae species commonly used as a diet for the culture of copepods, *Rhodomonas sp.* is often reported as being effective as a sole diet or as part of a mixed algal diet for *Acartia* species (Støttrup and Jensen, 1990; McKinnon et al., 2003; Knuckey et al., 2005; Morehead et al., 2005; Holste and Peck, 2006; Peck and Holste, 2006). The heterotrophic protists *Oxyrrhis marina* also proved to be an excellent choice for the *A.grani* diet, since these copepods are mainly suspension feeders and *O.marina* is present in the water column, unlike phytoplankton that after some hours tends to settle in the bottom. Another benefit of using *O.marina* as a component of the diets is the high quality of the water obtained. The remainder microalgae that were not eaten by copepods seemed to be consumed by these heterotrophic protists.

By all the above mentioned we suggest further studies on the use of a tri-algal combination, using *Oxyrrhis marina* and the two microalgae *Rhodomonas marina* and the Tahitian strain *Isochrysis sp.*

Copepods represent a nutritionally adequate feed for many larval fish species, and the data on biochemical composition may therefore serve as a base for nutritional improvements of enrichment media used in culture of intensive produced live feed for marine fish larvae, as well as for nutritional optimization of early weaning formulated diets.

Copepods fed an adequate diet are rich in highly unsaturated fatty acids including Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA), which are important for larval development (Ajiboye 2011, Shields et al. 1999). Dietary intake of these fatty acids is correlated with growth and development in copepods (Koski et al. 1998; Lacoste et al. 2001; Tang et al. 2001). Moreover, egg production, as mentioned before, is one of the principal factors determining copepod culture productivity and has been linked to the maternal nutrition (Castro-Longoria, 2003), especially n-3 polyunsaturated fatty acids (PUFAs), such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) (Milione and Zeng, 2007). EPA and DHA are synthesized de novo almost exclusively by primary producers while heterotrophs obtain them through bioaccumulation, or conversion of precursor fatty acids such as α-linolenic acid [18:3(n-3)].

Acartia grani adults fed with two of our best diets *Rhodomonas marina* and *Rhodomonas marina* + *Oxhyrris marina* presented moderate levels of lipids, with PUFAs accounting between 40-60% of total fatty acids detected. In comparison to other commonly fed zooplankton, copepods have superior nutritional content (Drillet et al. 2011, Støttrup 2000). Zooplankton species such as rotifers and *Artemia sp.* are more often the food provided for marine fish larvae; but unlike copepods they alone are considered nutritionally inadequate and must be enriched with additional nutrients before being used as feeds (Støttrup 2000). DHA levels found in our study were higher than those found in enriched *Artemia* with DHA-rich emulsion (Evjemo et al., 1997) and *Artemia* enriched with Super Selco (McEvoy et al., 1998). The amount of 20:4n-6 (ARA), which is a precursor for eicosanoid synthesis, was generally low (0.008-0.012 mg g^{-1}), giving an EPA/ARA range between 7.01and 24.89. Results found were in agreement to what Støttrup (1999) found when feeding Acartia tonsa with *Rhodomonas baltica*.

From an aquaculture standpoint, there is concern that the practice of using monoalgal diets as the sole diet for copepods reared in intensive systems may lead to deterioration in the nutritional value of the copepod (Støttrup and Jensen, 1990). Based on the above said, tough no significant differences were found on copepod composition between the two diets we recommend the use of *O. marina*, not only because it may improve water quality, by eating remains of microalgae, but also because, it has been suggested that heterotrophic protists may be able to synthesize or accumulate essential fatty acids (Kleppel et al. 1998), or convert precursor fatty acids to EPA and DHA (Broglio et al. 2003; Park et al. 2003).

A major problem in producing copepods is the inability to tolerate high densities. These densities are associated with cannibalism that is known to be common in *Acartia spp.* (Mauchline, 1998; McKinnon et al., 2003). In chapter VII results show that regardless

of the existence of food available in water column, the mortality of nauplii increase with the increased stocking density. The stocking density of 125ind./L was the treatment were the lowest cannibalism rate was observed. Small densities avoid the high rates of cannibalism and allowing the growth of the culture.

This work with *A.grani* does not end here. On the contrary, it opens a new series of questions that only further research can be answered. Shall be the diets used the optimal for production? Was the 38psu the better salinity? Are there interaction factors between abiotic and biotic variables? These are some of the questions among many others that only new research can answer. Another interesting experiment to be done would be to investigate the effectiveness of culturing *Acartia grani* as live feed for larvae fish. Moreover, it would be crucial to explore the possibility of producing *Acartia grani* in larval tanks, simulating mesocosms techniques.

4.2 Final Remarks

This thesis has brought some conclusions to improve the production of *Acartia grani.* We generally recommend that the incubation of eggs of *Acartia grani* for culturing purposes on the initial 2 days should be done at 28°C in order to improve egg hatching success. From that day onwards, temperature must be regulated for 24°C to 26°C, with a photoperiod of 12L:12D and copepods should be fed a diet composed by the cryptophyta microalgae *Rhodomonas sp.* and the heterotrophic protists *Oxyrrhis marina*.

I hope this research can be useful to scientists interested in copepods and for aquaculture in general.

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Annexes

Fig. 1- Mineral composition of the nutrient use in the culture of microalgas – Nutribloom (Necton).